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Expression of cyclooxygenase-2 related to angiogenesis in uterine cervical cancers

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Summary

Angiogenesis is essential for development, growth and advancement of solid tumors. Cyclooxygenase (COX)-2 is recognized as an angiogenic factor in various tumors. This prompted us to study the clinical implications of COX-2 expression related to angiogenesis in uterine cervical cancers. There was a significant correlation between microvessel counts and COX-2 levels in uterine cervical cancers. COX-2 localized in the cancer cells, but not in the stromal cells of uterine cervical cancer tissues. COX-2 levels increased with advancement, and the prognosis of the 30 patients with high COX-2 expression in uterine cervical cancers was poor (60%), while the 24-month survival rate of the other 30 patients with low COX-2 expression was 90%. Furthermore, COX-2 levels significantly correlated with VEGF levels in uterine cervical cancers. VEGF associated with COX-2 might work on angiogenesis in advancement. Therefore, long-term administration of COX-2 inhibitors might be effective on the suppression of regrowth or recurrence after intensive treatment for advanced uterine cervical cancers.

Introduction

Angiogenesis is essential for development, growth and advancement of solid tumors [1]. Angiogenic factors from tumors induce and activate matrix metalloproteinase, plasminogen activator, collagenase, and other enzymes in endothelial cells. The enzymes dissolve basement membrane of endothelial cells, after which the endothelial cells proliferate and migrate under the influence of angiogenic factors. Angiogenic factors induce production of integrins in the endothelial cells. The endothelial cells then form immature capillary tubes. Specific angiogenic factors produce specific angiogenesis in each tumor. The angiogenic factors vascular endothelial growth factor (VEGF) [2–6], thymidine phosphorylase (TP) identified with platelet-derived endothelial cell growth factor [7–11], interleukin (IL)-8 [12, 13], and basic fibroblast growth factor (bFGF) [14–16] along with the angiogenic transcription factor ETS-1 [17–20] work on angiogenesis in gynecological cancers, especially in uterine cervical cancer [2, 3, 7–9, 12, 14, 17] as follows.

The expression of VEGF was seen dominantly in the cancer cells, and correlated with microvessel density in uterine cervical cancers. Among the four subtypes of VEGF, the populations of $VEGF_{165}$ and $VEGF_{121}$ were dominant in normal uterine cervices and uterine cervical cancers. The levels of VEGF and $VEGF_{165}$ and $VEGF_{121}$ mRNAs were remarkably higher in some stage II and III/IV adenocarcinomas of the cervix than in other cases, including normal cervices. Therefore, the elevation of $VEGF_{165}$ and $VEGF_{121}$ might contribute to the

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relatively late advancement via angiogenic activity in some adenocarcinomas of the cervix [2]. The prognosis of patients with high TP in uterine cervical cancers was worse than those with low-TP cancers, and TP expression correlated with cellular proliferation and with vascular density and venous invasion in uterine cervical cancers. Therefore, TP might contribute to the growth of uterine cervical cancers via angiogenesis related to vascular spreading. Accordingly, TP in uterine cervical cancers might play a role of basic angiogenesis in all processes of advancement of uterine cervical cancers [7]. The TP level was increased in 8 of 40 metastatic lymph node lesions of uterine cervical cancers. The prognosis of the eight patients with high TP in metastatic lymph node lesions was extremely poor. On the other hand, the 24-month survival rate of the 32 patients with low TP in metastatic lymph node lesions was 75%. This indicates that TP contributes to the advancement of metastatic lesions, and that the TP level in metastatic lesions is a prognostic indicator [8]. IL-8 levels correlated with microvessel counts and infiltrated macrophage counts in uterine cervical cancers. Immunohistochemical staining revealed that the localization of IL-8 was similar to that of CD68 for macrophages. The prognosis of the 20 patients with high IL-8 in uterine cervical cancers was extremely poor, whereas the 24-month survival rate of the other 60 patients with low IL-8 was 67%. Therefore, this indicates that IL-8 is a prognostic indicator as an angiogenic factor supplied from macrophages within and around the tumor [12]. Basic FGF levels were higher in advanced primary uterine cervical cancers, regardless of histological type. Hence, this status contributes to the acceleration of growth, invasion, and metastasis with angiogenesis in advanced uterine cervical cancers [14]. Ets-1 gene expression levels correlated with microvessel counts in uterine cervical cancers. Immunohistochemical staining revealed that the localization of ETS-1 was similar to that of vascular endothelial cells. The level of ets-1 mRNA correlated with TP and IL-8 levels among angiogenic factors. Furthermore, the prognosis of the 25 patients with high ets-1 mRNA expression in uterine cervical cancers was extremely poor, while the 24-month survival rate of the other 25 patients with low ets-1 mRNA expression was 92%. Therefore, ETS-1 is a prognostic indicator as an angiogenic mediator in uterine cervical cancers [17].

Recently, it has been reported that COX-2 works on angiogenesis associated with tumor growth and advancement in the following tumors: advanced ovarian serous carcinomas [21], uterine endometrial cancers [22], breast cancers [23], gastric cancers [24], renal cell carcinomas [25], head and neck squamous cell carcinoma [26] and colon cancers [27]. Colon cancer cell line HCA-7 cells overexpress COX-2 and co-express VEGF, bFGF, transforming growth factor (TGF)-beta and platelet-derived growth factor (PDGF) [27]. The metabolites of COX-2, prostaglandin (PG)E1 and PGE2, have weak angiogenic activity [28], and PGE1 and PGE2 induce VEGF production in osteoblasts, synovial fibroblasts and macrophages [29–31]. This prompted us to study the protein expressions of COX-2 and various angiogenic factors, along with the various clinical backgrounds of uterine cervical cancer patients, to know the clinical implications of COX-2 in various uterine cervical cancers.

Materials and methods

Patients

Informed consent for the following studies was obtained from all patients and the Research Committee for Human Subjects, Gifu University School of Medicine. Sixty patients ranging from 26 to 85 years of age with uterine cervical cancers (25 stage-I cases, 19 stage-II cases, 16 stage-III cases; and 46 squamous cell carcinomas, 6 adenocarcinomas, 8 adenosquamous cell carcinomas) underwent curative surgery at the Department of Obstetrics and Gynecology, Gifu University School of Medicine, between December 2000 and January 2003. None of the patients had received any therapy before uterine cervical cancer tissue was taken. A part of each tissue of uterine cervical cancers was snap-frozen in liquid nitrogen to determine COX-2, IL-1α, IL-1β, TNF-α, IL-8, bFGF, VEGF and TP levels, and a neighboring part of the tissues was submitted for histopathological study including immunohistochemical staining for factor VIII-related antigen, CD34 and COX-2. The clinical stage of uterine cervical cancers was determined by International Federation of Obstetrics and Gynecology (FIGO) classification [32].

Immunohistochemistry

Four micrometer sections of formalin-fixed paraffin-embedded tissues of uterine cervical cancers were cut with a microtome and dried overnight at 37 °C on a silanized-slide (Dako, Carpinteria, CA, USA). Samples were deparaffinized in xylene at room temperature for 80 min and washed with a graded ethanol/water mixture and then with distilled water. Immunohistochemical staining for factor VIII-related antigen, which is synthesized by vascular endothelial cells, is specific for the endothelial cells of blood vessels [33], and is useful for detecting tumor angiogenesis [34]. The samples for COX-2 antigen were soaked in a citrate buffer, and then microwaved at 100 °C for 10 min, and those for factor VIII-related antigen and CD34 were treated with 0.3 μg/ml trypsin in phosphate buffer at room temperature for 20 min. The protocol for a DAKO LSAB2 Kit, Peroxidase (Dako) was followed for each sample. In the described procedures, rabbit anti-human COX-2 (Cayman Chemical, Ann Arbor, MI, USA), rabbit anti-factor VIII-related antigen (Zymed, San Francisco, CA, USA) and mouse CD34 (Dako) were used at dilutions of 1:25, 1:2, and 1:40, respectively, as the first antibodies. The addition of the first antibody, rabbit anti-human COX-2, rabbit anti-factor VIII-related antigen or mouse CD34, was omitted in the protocols for negative controls of COX-2, factor VIII-related antigen and CD34, respectively.

Immunohistochemistry was analyzed with three blocks from each patient, and three sections from each tissue block. The results of immunohistochemical staining for COX-2 were semiquantitatively evaluated as described by McCarty et al [35]. Each stained section was given a histochemical score (histoscore, HS) calculated by the formula: $\Sigma\ (i + 1) \times$ Pi, in which i = nuclear staining intensity (range 1–4, 0 indicates no staining) and Pi = percentage of stained cells. Vessels were counted in the five highest density areas at 200 × magnification (using a combination of 20 × objective and 10 × ocular, 0.785 mm^2 per field) by blinded investigators. Microvessel counts were expressed as the mean numbers of vessels in the five highest density areas [36]. Microvessel density was evaluated by the counting of microvessels.

Enzyme immunoassay for determination of COX-2, IL-1α, IL-1β, TNF-α, IL-8, bFGF, VEGF and TP antigens

All steps were carried out at 4 °C. Tissues of uterine cervical cancers (wet weight: 10–20 mg) were homogenized in HG buffer (5 mM Tris–HCl, pH 7.4, 5 mM NaCl, 1 mM $CaCl_2$, 2 mM ethyleneglycol-bis-[β-aminoethyl ether]-N,N,N′,N′-tetraacetic acid, 1 mM $MgCl_2$, 2 mM dithiothreitol, 25μg/ml aprotinin, and 25μg/ml leupeptin) with a Polytron homogenizer (Kinematics, Luzern, Switzerland). This suspension was centrifuged in a microfuge at 12,000 rpm for 3 min to obtain the supernatant. The protein concentration of samples was measured by the method of Bradford [37] to standardize COX-2, IL-1α, IL-1β, TNF-α, IL-8, bFGF, VEGF and TP antigen levels.

COX-2, IL-1α, IL-1β, TNF-α, IL-8, bFGF, and VEGF antigen levels in the samples were determined by a sandwich enzyme immunoassay using a Human COX-2 EIA Kit (Immuno-Biological Laboratories, Gunma, Japan), a Human IL-1α Quantikine (R & D System, Minneapolis, MN, USA), a Human IL-1β Quantikine (R & D System), a Human TNF-α Quantikine (R & D System), a Human IL-8 Quantikine (R & D System), a Human bFGF Quantikine (R & D System) and a Human VEGF Assay Kit-IBL (Immuno Biological Laboratories), respectively, and TP antigen levels were determined by the method described by Nishida, et al. [38]. The levels of COX-2, IL-1α, IL-1β, TNF-α, IL-8, bFGF, VEGF, and TP were standardized with the corresponding cellular protein concentrations.

Statistics

COX-2, IL-1α, IL-1β, TNF-α, IL-8, bFGF, VEGF, and TP levels were measured from three parts of the same tissue in triplicate. Statistical analysis was performed with Student's t-test. Differences were considered significant when p was less than 0.05. Correlation evaluations between microvessel counts and COX-2 levels and between VEGF and COX-2 levels were analyzed by Pearson's product-moment correlational coefficient. Positive correlation was considered significant when p was less than 0.05.

Results

There was a significant correlation between microvessel counts by immunohistochemical staining for factor VIII-related antigen (MVC-F8), and by that for CD34 (MVC-CD34) and COX-2 levels ($r = 0.614$, $p < 0.001$, and $r = 0.597$, $p < 0.001$, respectively) as shown in Figure 1, and also between MVC-F8/ MVC-CD34 and TP ($r = 0.659$, $p < 0.01/r = 0.591$, $p < 0.01$), between MVC-F8/MVC-CD34 and IL-8 ($r = 0.644$, $p < 0.01/r = 0.642$, $p < 0.01$), between MVC-F8/MVC-CD34 and VEGF levels ($r = 0.579$, $p < 0.01/r = 0.572$, $p < 0.01$) and between MVC-F8/MVC-CD34 and bFGF levels ($r = 0.566$, $p < 0.01/r = 0.598$, $p < 0.01$), but not between MVC and IL-1α, IL-1β or TNF-α levels (data not shown) in uterine cervical cancers. COX-2 diffusely localized in the cancer cells, but not in the stromal cells of ovarian cancer tissues in all cases given, as shown in Figure 2 for a representative case, a 34-year-old with a stage III, squamous cell carcinoma. Furthermore, COX-2 histoscores in stromal cells were extremely lower than those in cancer cells as shown in Figure 3, and COX-2 expression levels determined by ELISA were considered to be almost entirely supplied from cancer cells.

Among histopathological types, COX-2 expression did not demonstrate any specific relationship, as shown in Figure 4. COX-2 levels significantly increased with advancement (between stage I and stage II, $p < 0.0001$, between stage II and III, $p < 0.05$, and between stage I and stage III, $p < 0.0001$) as shown in Figure 5. The sixty patients were divided equally in two to form the low COX-2 and high COX-2 groups. The prognosis of the 30 patients with high COX-2 expression in uterine cervical cancers was poor (60%), while the 24-month survival rate of the other 30 patients with low COX-2 expression was significantly better (90%), as shown in Figure 6. Furthermore, COX-2 levels significantly ($r = 0.703$, $p < 0.001$) correlated with VEGF levels, as shown in Figure 7, but not with IL-1α, IL-1β, TNF-α, IL-8, bFGF or TP levels in uterine cervical cancers (data not shown).

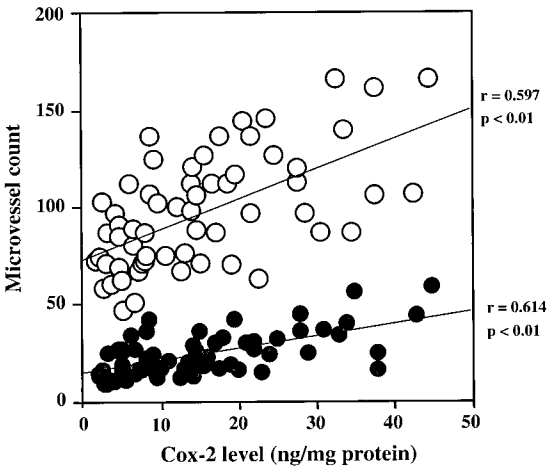

Figure 1. Correlation between microvessel counts and COX-2 in uterine cervical cancers Closed circles, microvessel counts by immunohistochemical staining for factor VIII-related antigen (MVC-F8); open circles, microvessel counts by immunohistochemical staining for CD34 (MVC-CD34).

Discussion

In immunohistochemistry, COX-2 overexpression can be used as a potent molecular risk factor in patients with stage IIB squamous cell carcinoma of the uterine cervix who are treated with radiotherapy and concurrent chemotherapy [39]. In surgically treated stage IB cervical cancer patients, COX-2 is expressed when lymph node or parametrial involvement is present, and COX-2 may downregulate apoptotic processes and thus enhance tumor invasion and metastasis [40]. Expression of COX-2 as determined by immunohistochemistry in adenocarcinoma of the cervix, 71%, was higher than that in squamous cell carcinoma, 29%, and increased COX-2 expression was concluded to play an important role in cervical adenocarcinomas [41]. COX-2 expression in cervical adenocarcinomas contributes to tumor progression by increasing cell proliferation as evaluated by immunohistochemistry for Ki-67 [42]. Furthermore, COX-2 was expressed in a greater proportion of adenocarcinoma patients than squamous cell carcinoma patients. COX-2 expression was also identified as a major determiner of a poor response to treatment and of an unfavorable prognosis, irrespective of the histologic type, reflecting the importance of the COX-2 protein in the acquisition of biologic aggressiveness and more malignant phenotype or increased resistance to the standard chemotherapy and radiotherapy in both histologic types [43]. The

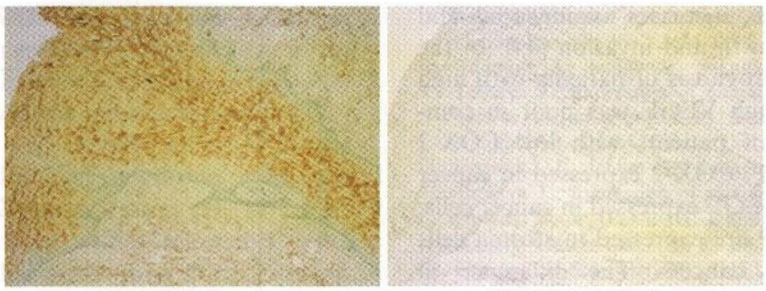

Figure 2. Immunohistochemical staining for COX-2 in uterine cervical cancers (original magnification, × 100). A representative case of squamous cell carcinoma of the uterine cervix. Rabbit anti-human COX-2 (Cayman Chemicals, Ann Arbor, MI, USA) was used at a dilution of 1:25 as the first antibody. Dark brown staining represents positive for COX-2 antigen.

protein expression assay by immunohistochemistry is semiquantitative. We evaluated absolute and quantitative COX-2 expressions by ELISA to avoid variation in evaluations by each individual immunohistochemistry. Therefore, some discrepancy could arise from different methods to determine COX-2 expression levels. In the present study, COX-2 levels increased with advancement, and increasing COX-2 was linked to poor prognosis, regardless of the histopathological type.

COX-2 expression in uterine cervical adenocarcinomas contributes to tumor progression by increasing microvessel density-evaluated angiogenesis as determined by immunohistochemistry for CD34 [42]. In the present study, microvessel counts by immunohistochemical staining for factor VIII-related antigen, and by that for CD34 correlated with COX-2, TP, IL-8 and VEGF levels in uterine cervical cancers. Therefore, COX-2, TP, IL-8 and VEGF may work on angiogenesis as angiogenic factors or mediators. The expression of COX-2 in adenoid cystic carcinoma induces the

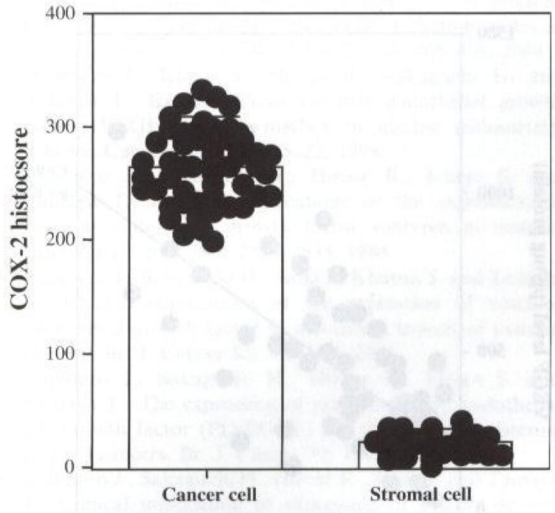

Figure 3. Histoscores between cancer and stromal cells of uterine cervical cancers Histoscores were calculated by the formula: $\Sigma\ (i + 1) \times$ Pi, in which i = nuclear staining intensity (range 1–4, 0 indicates no staining) and Pi = percentage of stained cells by immunohistochemistry for COX-2.

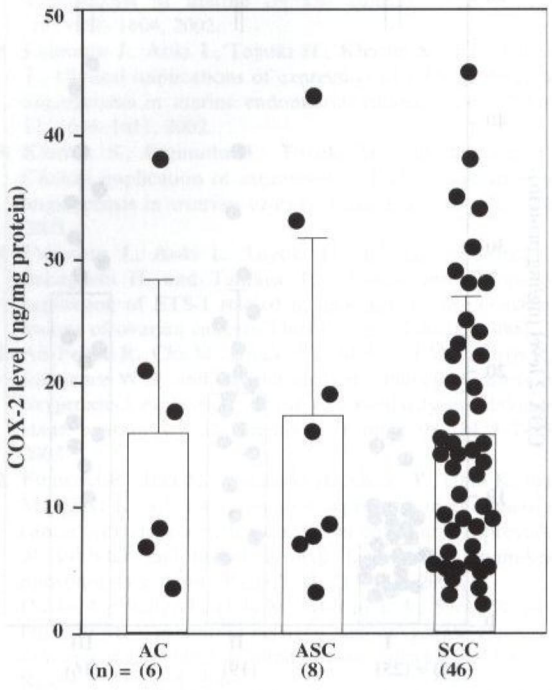

Figure 4. Levels of COX-2 in uterine cervical cancers classified according to histopathological types SCC, squamous cell carcinoma; AC, adenocarcinoma; ASCC, adenosquamous cell carcinoma. Each level is the mean of 9 determinations. The bars show SD.

expression of VEGF, increases angiogenesis and enhances tumor growth and invasion [44]. In the present study, the prognosis of patients with high COX-2 linked to high VEGF was poor in comparison with that of patients with low COX-2 linked to low VEGF. COX-2 expressed in cancer cells might induce VEGF expressed in cancer cells. IL-8, bFGF and TP are expressed in stroma cells of uterine cervical cancers. The difference in localization of angiogenic factors might cause COX-2 level to be proportional to VEGF level, but not other angiogenic factor levels, such as IL-8, bFGF and TP levels. Among angiogenic factors, VEGF has been evaluated as an important factor for tumor angiogenesis, which is essential for the growth of solid tumors. Generally speaking, VEGF secreted from tumors contributes to tumor growth not via an autocrine pathway to tumor cells, but via a paracrine pathway to surrounding microvessels [45]. Therefore, angiogenesis induced by VEGF associated with COX-2 might be essential to advancement linked to prognosis in uterine cervical cancers.

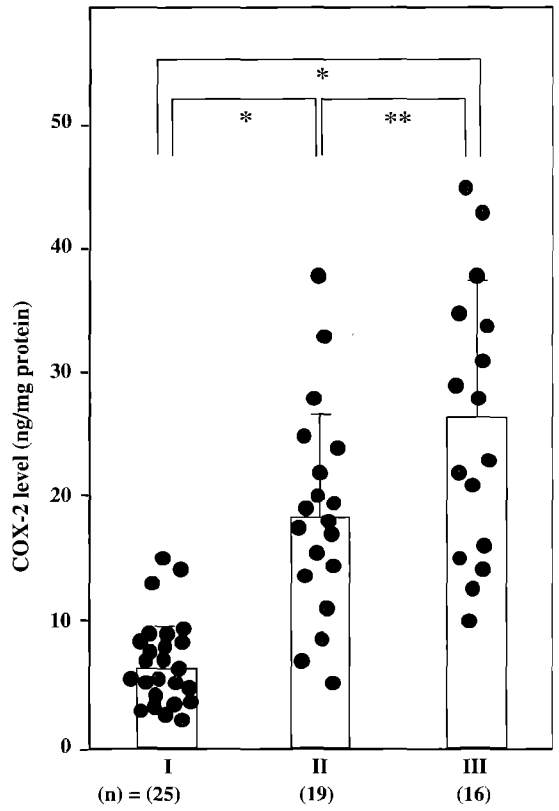

Figure 5. Levels of COX-2 in uterine cervical cancers classified according to clinical stages Clinical staging of uterine cervical cancer was done according to FIGO. Each level is the mean of 9 determinations. The bars show SD $*p < 0.0001$, $**p < 0.05$.

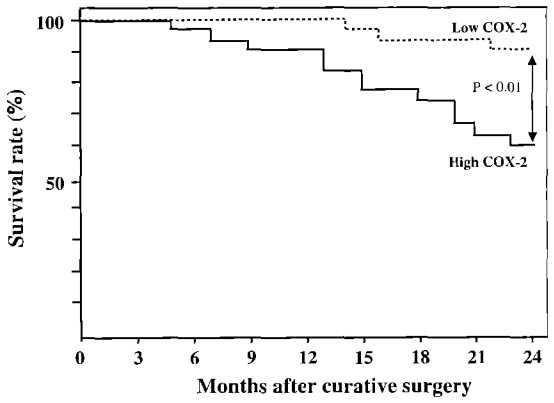

Figure 6. Survival rate after curative resection for uterine cervical cancers Patient prognosis was analyzed with a 24-month survival rate. High COX-2, cases with high COX-2 levels (> 14 ng/mg protein), $n = 30$; Low COX-2, cases with low COX-2 levels (< 14 ng/mg protein), $n = 30$.

Based on the present study, it is strongly suggested that inhibition of COX-2 might lead to suppression of VEGF expression in uterine cervical cancers. Both endothelial migration, and tube formation stimulated by COX-2-overexpressing cells are inhibited by COX-2 antibodies to combinations of angiogenic factors, by NS-398 (a selective COX-2 inhibitor), and by aspirin [27].

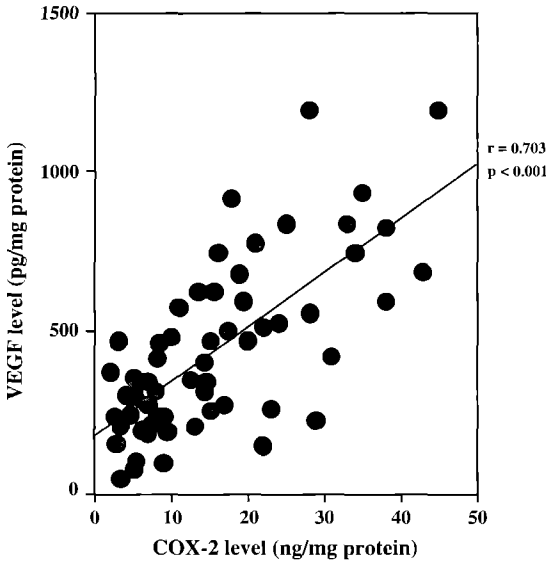

Figure 7. Correlation between COX-2 and VEGF in uterine cervical cancers.

Furthermore, the COX inhibitor ibuprofen has already been shown to inhibit angiogenesis in rat glioma [46]. A selective COX-2 inhibitor (NS-398) and aspirin inhibited the production of VEGF, bFGF, TGF-beta and PDGF along with the stimulation of endothelial migration and tube formation in COX-2-overexpressing cells [27]. Furthermore, the treatment with a selective COX-2 inhibitor celecoxib decreases tumor COX-2 expression and markers of proliferation and neoangiogenesis in uterine cervical cancers [47]. Therefore, long-term administration of COX-2 inhibitors might be effective for the suppression of advancement and of regrowth or recurrence after intensive treatment for advanced uterine cervical cancers, regardless of the histopathological type.

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