Elevated Cyclooxygenase-2 Expression Is Associated with Altered Expression of p53 and SMAD4, Amplification of HER-2/neu, and Poor Outcome in Serous Ovarian Carcinoma

Tiina-Liisa Erkinheimo,[1,5] Heini Lassus,[1] Patrik Finne,[3] Bastiaan P. van Rees,[6] Arto Leminen,[1] Olavi Ylikorkala,[1] Caj Haglund,[4] Ralf Butzow,[1,2] and Ari Ristimäki[2,5]

Departments of [1]Obstetrics and Gynecology, [2]Pathology, [3]Clinical Chemistry, and [4]Surgery, Helsinki University Central Hospital and [5]Molecular and Cancer Biology Research Program, Biomedicum Helsinki, University of Helsinki, Helsinki, Finland, and [6]Department of Pathology, Academic Medical Center, Amsterdam, the Netherlands

ABSTRACT

Purpose and Experimental Design: **Cyclooxygenase-2 (COX-2) is frequently expressed in human adenocarcinomas and inhibition of COX-2 suppresses tumor formation in various animal models of carcinogenesis. We analyzed expression of COX-2 protein in human serous ovarian carcinomas by immunohistochemistry ($n = 442$) and by Western blotting ($n = 12$) and COX-2 mRNA by reverse transcriptase PCR ($n = 12$). COX-2 immunoreactivity was correlated to clinicopathological variables and to expression of p53 and SMAD4 as detected by immunohistochemistry and to amplification of HER-2/neu as detected by *in situ* hybridization.**

Results: **COX-2 mRNA expression was detected in 75% (9 of 12) and COX-2 protein in 42% (5 of 12) of the serous ovarian adenocarcinoma specimens as detected by reverse transcriptase-PCR and Western blot analysis, respectively. Moderate to strong (elevated) immunoreactivity for COX-2 was detected in 70% (310 of 442) of the tumors. Elevated COX-2 expression associated with reduced disease-specific survival ($P = 0.0011$), high histological grade ($P < 0.0001$), residual tumor size > 1 cm ($P = 0.0111$), and age > 57 years ($P = 0.0099$). Tumors with altered immunostaining pattern for p53 or SMAD4 expressed more frequently elevated levels of COX-2 when compared with the tumors with normal staining pattern of these tumor suppressor genes ($P < 0.0001$ and $P = 0.0004$, respectively). In addition, elevated COX-2 expression associated with amplification of HER-2/neu oncogene ($P = 0.0479$).**

Conclusions: **Our results suggest that elevated expression of COX-2 associates with reduced survival in serous ovarian carcinomas and that expression of COX-2 may be induced in these tumors by loss of tumor suppressor genes such as *p53* and *SMAD4* and by amplification of *HER-2/neu* oncogene.**

INTRODUCTION

Expression of cyclooxygenase-2 (COX-2) is elevated in a variety of human malignancies and in their precursor lesions (1–3). Inhibition of COX-2 activity or its genetic deletion leads to reduced number and size of intestinal polyps in mouse models of familial adenomatous polyposis (4, 5), and overexpression of COX-2 promoted mammary gland tumorigenesis in a transgenic mouse model (6). Furthermore, a COX-2-selective drug, celecoxib, reduced polyp burden in patients who suffer from familial adenomatous polyposis (7). Thus, COX-2 constitutes a rational target in chemoprevention.

Ovarian carcinoma exhibits several histological subtypes with different pathogenesis and outcome. Of the ovarian carcinomas, serous type is the most common, comprising >50% of the cases, and it has a relatively poor prognosis the 5-year survival being ~40% (8, 9). The aggressive nature of this disease is in accordance with the presence of higher frequency of cytogenetic changes than in other types of ovarian carcinoma (10, 11). The molecular pathogenesis of serous ovarian carcinoma is largely unknown, but altered expression of tumor suppressor gene *p53* is frequent and confers poor prognosis (12–14). Furthermore, distal half of chromosome 18q is among the most frequently lost genomic regions in serous ovarian carcinoma (15–17). Interestingly, expression of tumor suppressor gene *SMAD4*, located at 18q21, is reduced in 28% of serous ovarian carcinomas (17). In respect of oncogenes, amplification or overexpression of *HER-2/neu* is found in ~30% of ovarian carcinomas, and it is associated with poor prognosis (18, 19).

It was recently reported that expression of COX-2 is elevated in ovarian cancer (20, 21). Moreover, elevated COX-2 expression has been reported to be an independent prognostic factor in ovarian cancer but that it did not associate with any other clinicopathological parameter, such as stage and grade (22). We have now studied expression of COX-2 protein in 442 serous ovarian carcinoma specimens using immunohistochemistry and have correlated the expression to prognosis, to clinicopathological parameters, and to expression of tumor suppressor genes *p53* and *SMAD4* and amplification of *HER-2/neu* oncogene. Our results show that elevated COX-2 expression correlates with reduced survival in patients who suffer from

Received 1/28/03; revised 8/15/03; accepted 9/24/03.
Grant support: The Academy of Finland, the Finnish Cancer Foundation, the Helsinki University Central Hospital Research Funds, the Sigrid Jusélius Foundation, and Merck Medical School Program. T-L. E. was supported by the Finnish Cultural Foundation.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Requests for reprints: Ari Ristimäki, Molecular and Cancer Biology Research Program, Biomedicum Helsinki, Room B512b, University of Helsinki, P. O. Box 63 (Haartmaninkatu 8), FIN-00014 Helsinki, Finland. Phone: 358-9-191-25588; Fax: 358-9-191-26700; E-mail: Ari.Ristimaki@hus.fi.

serous ovarian adenocarcinoma and that increased expression of COX-2 may depend on loss of tumor suppressor gene activity and increase in oncogene activity.

MATERIALS AND METHODS

Patients. The study consisted of 474 consecutive patients treated for serous ovarian carcinoma at the Department of Obstetrics and Gynecology of Helsinki University Central Hospital between the years 1964 and 2000, and whose specimens could be retrieved from the pathology files. The study was approved by the local ethics committee. The histology was determined by a gynecological pathologists and was verified by another pathologist (R. B.). The clinical information of the patients was extracted from the patient records and survival information from the Population Register Center. The median age of ovarian cancer patients was 57 years (range, 15–88 years) at the time of diagnosis. The median follow-up time of patients alive at the end of study period was 5.2 years (range, 0.4–36.1 years). The patients were operated by gynecological oncologists, and the clinical staging and grading were performed according to Federation of International Gynecology and Obstetrics. New treatment regimens were adopted as follows: platinum-based chemotherapy at the beginning of the 1980s; radical surgery at the end of the 1980s; and paclitaxel/platinum chemotherapy after 1996. The tumor samples for the study were obtained at primary surgery before patients had received any chemotherapy.

Microarray Analysis. Tissue microarrays were constructed as described previously (23). In brief, tumor tissues were embedded in paraffin, and 5-μm sections were cut from each block and stained with H&E. A representative tumor area was selected from H&E-stained sections of each tumor by our pathologist (R. B.). Four tissue cores (diameter, 0.8 mm) were obtained from each tumor block and replaced into recipient paraffin block with a custom-built precision instrument (Beecher Instruments, Silver Spring, MD). These samples were cut with a microtome into 5-μm sections. The presence of cancer cells was verified on H&E-stained sections.

Immunohistochemistry and *in Situ* Hybridization. Specificity of the antibody and detailed immunohistochemistry protocol have been described previously (24, 25). Specimens were deparaffinized and antigen retrieved using microwave oven [(4 × 5 min in 700 W in 0.01 M sodium citrate buffer (pH 6)]. The slides were then immersed in 0.6% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity and in blocking solution (1.5:100 normal horse serum in PBS) for 15 min to block unspecific binding sites. Immunostaining was performed with a COX-2-specific antihuman monoclonal antibody (160112; Cayman Chemical Co., Ann Arbor, MI) in a dilution of 1:200 (2.5 μg/ml) in PBS containing 0.1% sodium azide and 0.5% BSA at room temperature for overnight. The sections were then treated with biotinylated horse antimouse immunoglobulin (1:200; Vector Laboratories, Inc., Burlingame, CA) and avidin-biotin peroxidase complex (Vectastain ABComplex; Vector Laboratories, Inc.). The peroxidase staining was visualized with 3-amino-9-ethylcarbazole (Sigma Chemical Co., St. Louis, MO), and the sections were counterstained with Mayer's hematoxylin. To confirm the specificity of the staining, 12 histological sections with serous ovarian cancer and 6 non-neoplastic ovarian samples were stained with and without human COX-2 control peptide (20 μg/ml; Cayman Chemical Co.) for 1 h at room temperature before the staining procedure. Immunostaining for p53 ($n = 437$) and SMAD4 ($n = 433$) of the serous ovarian carcinomas has been described previously (17). HER-2/neu gene amplification was assessed using chromogenic *in situ* hybridization ($n = 323$) according to the method of Tanner *et al.* (26).

Scoring. The intensity of staining was scored independently and in blinded manner by three investigators (T-L. E., B. v. R., and A. R.) from 474 serous ovarian carcinoma tissue cores on the following scale; 0, no staining; 1, weak diffuse cytoplasmic staining (may contain stronger intensity in <10% of the cancer cells); 2, moderate granular cytoplasmic staining in >10% of the cancer cells; and 3, strong granular cytoplasmic staining in >50% of cancer cells. Only those tumors that exhibited at least three tissue cores were included to the analysis (442 of 474; 93%). All specimens with discordant scores were reevaluated using a multiheaded microscope, and the consensus score was used for additional analyses.

The immunoreactivity for p53 was scored according to the intensity of nuclear staining (negative, weak, and moderate or strong) and to the percentage of positively stained tumor cells (<20, 20–49, and ≥50%). Tumors with weak immunostaining, similar to that found in the surface epithelium of normal ovaries and in normal serous epithelium of the fallopian tube, were regarded as showing normal p53 expression. The majority (94%) of these tumors had positive staining in <20% of tumor cells. Two distinct patterns of altered p53 expression were identified: completely negative p53 in which no staining was found in any of the tumor cells and excessive p53 in which >50% of tumor cells showed moderate or strong immunopositivity (44). Immunostaining for SMAD4 was scored altered whenever the signal was absent or very weak, which was different from the staining observed in the surface epithelium of normal ovaries and the general pattern of positive staining of the tumor samples (17).

Western Blot Analysis. Tumor samples (50 mg) of 12 serous ovarian carcinoma specimens were crushed in 1 ml of radioimmunoprecipitation assay buffer [150 mM NaCl, 1% NP40, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, and 50 mM Tris (pH 8.0)] supplemented with Complete mini protease inhibitor mixture tablet (Boehringer, Mannheim, Germany) by Fast Prep homogenization instrument (Qbiogene, Inc., Carlsbad, CA) and centrifuged at 14,000 × *g* for 15 min. Protein concentration was measured with BSA protein assay (Pierce, Rockford, IL). Proteins (100 μg) were resuspended in sample loading buffer [74 mM Tris-HCl (pH 6.8), 2% SDS, 12% glycerol, 5% β-mercaptoethanol, and 0.015% bromphenol blue] and separated by SDS-PAGE (12%). The proteins were transferred electrophoretically to Hybond-C extra nitrocellulose membranes (Amersham, Buckinghamshire, United Kingdom). Nonspecific binding was blocked by TBS-NP40, 5% low-fat dry milk solution, overnight at 4°C. For immunodetection, the membrane was incubated with the mouse antihuman monoclonal COX-2 antibody (160112, 1:1000 dilution; Cayman Chemical Co.) with or without COX-2 blocking peptide (360107; Cayman Chemical Co.) for 1 h at room temperature. The membrane was then washed three times in TBS-NP40 and incubated with the sheep

antimouse antibodies conjugated to horseradish peroxidase (1:2000 dilution). After four washes with TBS-NP40, COX-2 was visualized by enhanced chemiluminescence with Super Signal West Femto Maximum Sensitivity Substrate (Pierce) according to the manufacture's protocol. Loading was controlled by goat antihuman β-actin antibody (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) with donkey antigoat antibodies conjugated to horseradish peroxidase (1:2000 dilution; Santa Cruz Biotechnology) as the second antibody. The signals were visualized using MultiImage FC Light Cabinet (Promega, Madison, MI) and the digital imaging FluorChem 8800 software (α Innotech Corporation, San Leandro, CA).

RNA Isolation and Reverse Transcriptase-PCR. Tumor samples (100 mg) of 12 serous ovarian carcinoma specimens were crushed in 1 ml of TRIzol (Invitrogen, Carlsbad, CA) by Fast Prep instrument, after which RNA was purified by isopropanol precipitation after phenol/chloroform extraction. RNA concentration was determined by absorbance at 260 nm. Total RNA (1 μg) from 12 serous ovarian cancer samples was converted to cDNA with Moloney murine leukemia virus reverse transcriptase RNase H minus, RNasin (Promega), 2′deoxynucleoside 5′triphosphates (Pharmacia Biotech), and random primers (Invitrogen) in a volume of 50 μl. The reverse transcriptase reaction mix of 5 μl was PCR amplified in reaction mixture of 45 μl that contained 2 units of Dynazyme II polymerase and Dynazyme buffer (Finnzymes, Espoo, Finland) and antisense and sense primers for COX-2 (0.5 μg) or glyseraldehyde-3-phosphate dehydrogenase (0.15 μg). The nucleotide sequences of the primer for COX-2 are as follows: 5′-TTCAAATGAGATTGTGGGAAAAT-3′ (sense) and 5′-AGATCATCTCTGCCTGAGTATCTT-3′ (antisense); and for glyseraldehyde-3-phosphate dehydrogenase: 5′-CCACCCATGGCAAATTCCATGGCA-3′ (sense) and 5′-TCTAGACGGCAGGTCAGGTCCACC-3′ (antisense; Ref. 27). The PCR reaction mixture was heated to 94°C for 3 min followed by amplification for COX-2 by 40 and for glyseraldehyde-3-phosphate dehydrogenase by 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. After the last cycle, the reactions were remained at 72°C for an additional 15 min. Amplified cDNAs were analyzed by 1.2% agarose gel electrophoresis and ethidium bromide staining. The amplified products were visualized under UV transillumination in MultiImage FC Light Cabinet by using the digital imaging FluorChem 8800 software.

Statistical Analysis. For statistical analysis, COX-2 scores 0 and 1 were combined (low COX-2 expression), and scores 2 and 3 represented elevated COX-2 expression. The correlation between COX-2 staining intensity and clinically relevant and prognostic variables was assessed by using the χ^2 test or by Fisher's exact test. Probability of survival was estimated using the Kaplan-Meier method. Survival probabilities were compared between groups using the log-rank test or log-rank test for trend. Disease-specific survival time was defined as the time from primary surgery to death of the patient from ovarian cancer or to the end of the follow-up. Of the 442 patients with known COX-2 score, 244 (55%) died from ovarian cancer, 5 (1%) because of another cancer, and 32 (7%) because of another disease. The 37 deaths due to other causes than ovarian cancer were treated as censored cases, as well as those patients who were alive at the end of the follow-up ($n = 161$). Multivariate survival analysis was performed using the COX proportional hazards model, entering the following variables: COX-2 expression (score 0–1 *versus* 2–3); age (≤57 *versus* >57 years), grade (1, 2, and 3); stage (I, II, III, and IV); tumor size (≤10 *versus* >10 cm); and residual tumor size (≤1 *versus* >1 cm). All these data were available from 383 patients.

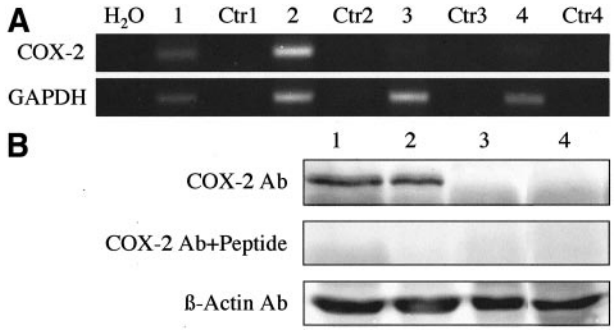

Fig. 1 Cyclooxygenase-2 (COX-2) expression in serous ovarian adenocarcinoma specimens. *A*, COX-2 mRNA was expressed in 9 of 12 tumor samples as detected by reverse transcriptase-PCR. Two positive (samples 1 and 2) and two negative ones (samples 3 and 4) are shown. Control lane without cDNA (H_2O) and reverse transcriptase controls (Ctr1–4) are also shown. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. *B*, COX-2 protein expression of the same samples (1–4) was detected by Western blot analysis. COX-2 signal was totally blocked by preadsorption with the antigenic peptide as shown in the *middle panel*. β-Actin served as a loading control.

RESULTS

COX-2 Expression in Serous Ovarian Cancer Specimens as Detected by Reverse Transcriptase-PCR, Western Blot Analysis, and Immunohistochemistry. Expression of COX-2 mRNA was detected in 75% (9 of 12) and COX-2 protein in 42% (5 of 12) of the serous ovarian adenocarcinoma specimens as detected by reverse transcriptase-PCR and Western blot analysis, respectively. All tumors that expressed COX-2 protein were also positive for COX-2 mRNA. In Fig. 1, two positive specimens and two reverse transcriptase-PCR-negative samples are shown. The specificity of the antibody was confirmed by preadsorption with the antigenic peptide. This blocking procedure abolished the signal for COX-2 protein signal, thus confirming the specificity of the antibody (*middle panel* in Fig. 1*B*). Expression of COX-2 protein was analyzed in 442 serous ovarian carcinoma specimens using immunohistochemistry, of which 1% ($n = 3$) were negative (score 0) and 29% ($n = 129$) weakly (score 1), 48% ($n = 211$) moderately (score 2), and 22% ($n = 99$) strongly (score 3) positive. Moderate and strong (scores 2 and 3) cytoplasmic granular COX-2 immunoreactivity were defined as elevated expression and was seen in 70% of the tumors. Expression of COX-2 protein localized to the cytoplasm and to the perinuclear region of the cancer cells (Fig. 2), but stromal compartment was positive in only 7.9% ($n = 35$) of the tumors. The ovarian surface epithelium was negative or only weakly positive for COX-2 staining (Fig. 2).

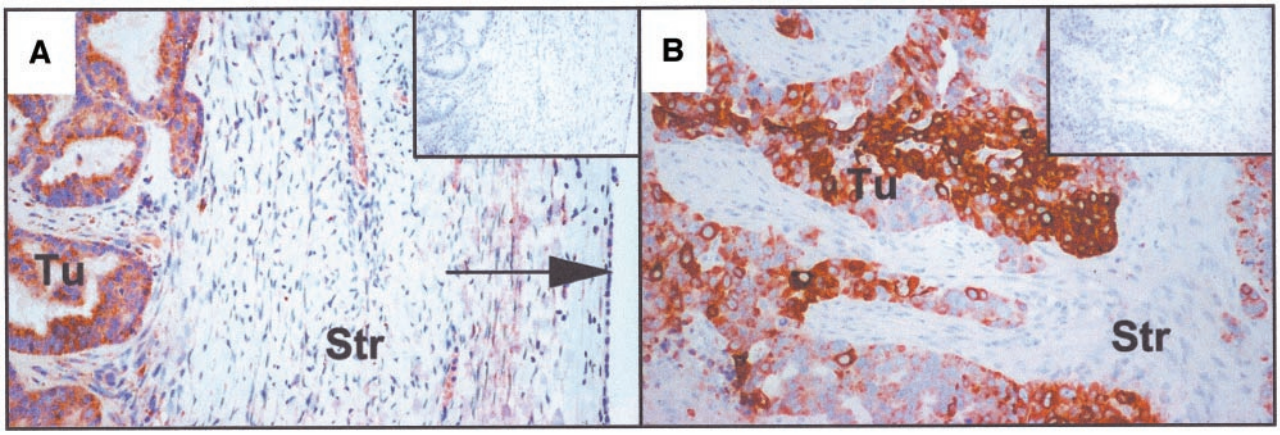

Fig. 2 Cyclooxygenase-2 (COX-2) protein expression in serous ovarian adenocarcinoma specimens as detected by immunohistochemistry. *A*, moderate immunoreactivity (score 2) in serous ovarian carcinoma (stage I, grade 1). Neoplastic tumor cells were positive (Tu), whereas nonneoplastic stroma (Str) and the lining nonneoplastic epithelium (*arrow*) were negative or only weakly positive. Original magnification, ×200. *B*, strong immunoreactivity (score 3) in serous ovarian carcinoma (stage III, grade 3). Original magnification, ×400. For both figures the antigen blocking control is shown in the *insert*.

Table 1 Association of elevated cyclooxygenase-2 (COX-2) immunoreactivity with clinicopathological parameters in serous ovarian adenocarcinoma

Clinical features	*n* (total)	Elevated COX-2/*n* (%)	*P*[a]
Age (yrs)	442		=0.0099
≤57		144/223 (65%)	
>57		166/219 (76%)	
Histological grade	436		<0.0001
1		96/166 (58%)	
2		86/114 (75%)	
3		125/156 (80%)	
Stage	440		NS[b] (=0.1753)
I		63/90 (70%)	
II		43/61 (70%)	
III		159/237 (67%)	
IV		43/52 (83%)	
Tumor size	436		NS (=0.3725)
≤10 cm		95/142 (67%)	
>10 cm		209/294 (71%)	
Residual tumor size	397		=0.0111
≤1 cm		126/195 (65%)	
>1 cm		154/202 (76%)	

[a] χ^2 test.
[b] Not significant.

Association of COX-2 Expression with Clinicopathological Parameters. Elevated expression of COX-2 associated with age > 57 years ($P = 0.0099$), high histological grade ($P < 0.0001$), and residual tumor size (maximal diameter of the tumor left in the patient at primary operation) > 1 cm ($P = 0.0111$). No correlation was found between COX-2 staining intensity and stage or size of the tumor (Table 1).

Association of COX-2 Expression with Disease-Specific Survival. Elevated COX-2 expression was associated with decreased disease-specific survival among the 442 serous ovarian cancer patients ($P = 0.0011$; Fig. 3*A*). When COX-2 expression was divided into three categories (score 0–1, score 2, and score 3), probability of survival was progressively reduced ($P = 0.0004$; Fig. 3*B*). In addition to COX-2, the variables that correlated with the disease-specific overall survival were age, histological grade, stage, tumor size, and residual tumor size (Table 2).

Multivariate Analysis. Multivariate analysis was performed to evaluate the independence of COX-2 expression as a

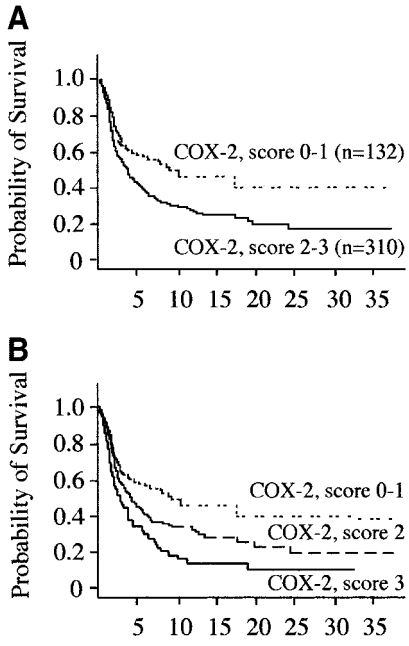

Fig. 3 Disease-specific survival of 442 patients with serous ovarian adenocarcinoma according to cyclooxygenase-2 (COX-2) protein expression as detected by immunohistochemistry. *A*, survival of patients with low (score 0–1) or with moderate to strong, *i.e.*, elevated (score 2–3) COX-2 expression (log-rank test, $P = 0.0011$). *B*, survival of patients with COX-2 staining intensity score 0–1 *versus* score 2 *versus* score 3 (log-rank test for trend, $P = 0.0004$).

Table 2 Five-year disease-specific survival of serous ovarian adenocarcinoma patients according to cyclooxygenase-2 (COX-2) immunoreactivity and to clinicopathological parameters

Clinical features	*n* (total)	n (%)	5-year survival (95% CI)[a]	*P*[b]
COX-2 expression	442			=0.0011
Low (score 0–1)		132 (30%)	59.6 (50.9–68.4)	
Elevated (score 2–3)		310 (70%)	44.6 (38.7–50.5)	
Age	474			<0.0001
≤57 years		238 (50%)	62.3 (55.9–68.7)	
>57 years		236 (50%)	35.8 (29.1–42.4)	
Histological grade	466			<0.0001
1		181 (39%)	80.8 (74.8–86.7)	
2		121 (26%)	35.5 (26.2–44.8)	
3		164 (35%)	23.1 (16.0–30.2)	
Stage	471			<0.0001
I		101 (21%)	88.6 (81.9–95.3)	
II		64 (14%)	62.8 (50.3–75.2)	
III		251 (53%)	38.9 (32.4–45.3)	
IV		55 (12%)	8.5 (0–17.2)	
Tumor size	467			=0.0220
≤10 cm		152 (33%)	54.5 (46.0–62.9)	
>10 cm		315 (67%)	46.9 (41.1–52.7)	
Residual tumor size	423			<0.0001
≤1 cm		207 (49%)	74.6 (68.1–81.0)	
>1 cm		216 (51%)	21.3 (15.4–27.1)	

[a] Confidence interval.
[b] Log-rank test or log-rank test for trend.

prognostic factor. In this analysis age ($P = 0.0134$), grade ($P < 0.0001$), stage ($P < 0.0001$), and residual tumor size ($P < 0.0001$) were identified as independent prognostic factors, but inclusion of tumor size ($P = 0.8989$) or COX-2 ($P = 0.7439$) did not add significant independent prognostic information.

Correlation of COX-2 Expression with Expression of p53 and SMAD4 and with Amplification of *HER-2/neu*. Elevated COX-2 expression was found more frequently in tumors with altered (completely negative or increased) p53 staining when compared with the tumors with normal (weak) p53 immunoreactivity (Table 3). Altered p53 staining was found in 38% (50 of 132) of COX-2 score 0–1, in 63% (130 of 208) of score 2, and in 81% (79 of 97) of score 3 ($P < 0.0001$). In respect of SMAD4, elevated COX-2 expression was also found more frequently in tumors with altered (negative or decreased) SMAD4 staining when compared with the tumors with normal SMAD4 immunostaining (Table 3). Altered SMAD4 staining was found in 22% (28 of 130) of COX-2 score 0–1, in 31% (63 of 206) of score 2, and in 58% (56 of 97) of score 3 ($P < 0.0001$). Tumors with combined

Table 3 Association of elevated cyclooxygenase-2 (COX-2) expression with immunostaining for p53 and SMAD4 in serous ovarian adenocarcinoma

Immunostaining	*n* (total)	Elevated COX-2/*n* (%)	*P*[a]
p53			
Normal	437	96/178 (54%)	<0.0001
Negative		59/67 (88%)	
Increased		150/192 (78%)	
SMAD4			
Normal	433	184/286 (64%)	=0.0004
Negative or decreased		119/147 (81%)	
Normal p53 and normal SMAD4		76/143 (53%)	
Normal p53 and altered SMAD4		18/31 (58%)	NS[b] (=0.69)[c]
Altered p53 and normal SMAD4		108/143 (76%)	=0.0001[c] NS (=0.07)[d]
Altered p53 and altered SMAD4		99/114 (87%)	<0.0001[c] =0.0013[d] =0.0264[e]
Normal HER-2	323	163/254 (64%)	=0.0479
Amplified HER-2		53/69 (77%)	

[a] Fisher's exact test.
[b] Not significant.
[c] *Versus* normal p53 and normal SMAD4.
[d] *Versus* normal p53 and altered (negative or decreased) SMAD4.
[e] *Versus* altered (negative or increased) p53 and normal SMAD4.

defective expression of p53 and SMAD4 expressed more frequently elevated levels of COX-2 when compared with the tumors with only one of the tumor suppressor genes showing altered expression (Table 3). Elevated expression of COX-2 was found more frequently in tumors with amplification of HER-2/neu when compared with the tumors with normal HER-2/neu status ($P = 0.0479$; Table 3).

DISCUSSION

Our results provide evidence for COX-2 protein expression in a large series of consecutive cases of serous ovarian cancer specimens ($n = 442$). Elevated COX-2 expression was found by immunohistochemistry in 70% of the tumors, and it associated with reduced disease-specific survival ($P = 0.0011$). In accord with our data, elevated COX-2 expression has previously been detected in ovarian cancer (20–22, 28–31). However, a relatively high variation of COX-2 positivity (42–89%) was observed in these studies, which may depend on the use of different antibodies, scoring criteria, and selection of the patients. Similarly to our data, Denkert *et al.* (22) reported recently that elevated COX-2 expression was a marker for poor prognosis in 86 invasive ovarian cancer patients of various histological types but that COX-2 did not correlate with any of the clinicopathological variables, including histological grade. Moreover, elevated COX-2 expression was identified as an independent prognostic factor, which is consistent with our finding in esophageal adenocarcinoma (32). However, we were unable to recognize COX-2 as an independent prognostic variable in the current ovarian cancer material but found instead a highly significant correlation of COX-2 expression with high histological grade, which was a strong independent covariate in the multivariate assay. There is no generally accepted grading system for ovarian carcinoma mainly because the same criteria are not applicable to all histological types. We used Federation of International Gynecology and Obstetrics grading system, which is the most widely used and which has proven to be a powerful determinant of patient outcome especially in serous tumors (8). This was also seen in our analysis. Denkert *et al.* (22) used a different grading system and included several histological subtypes in their material, which may explain the discordance in the findings between their and our materials.

Nonneoplastic ovarian surface epithelium expresses only low or nondetectable levels of COX-2 protein, but frequency of COX-2 positivity seems to increase when benign ovarian tumors (0–64%) were compared with tumors with low malignant potential (37–67%) or to invasive carcinomas (42–89%; Refs. 20–22). In addition to immunohistochemistry, we and others have detected COX-2 mRNA (22, 31) and the protein as detected by immunoblotting (29) in ovarian cancer specimens. We found expression of COX-2 mRNA in 9 of 12, Denkert *et al.* in 4 of 5 (22), and Shigemasa *et al.* in 26 of 36 (31) of ovarian adenocarcinoma specimens. Combined these data indicate that COX-2 mRNA is expressed in 72–80% of the ovarian carcinomas, which is consistent with our immunohistochemistry results (elevated expression in 70% of the tumors). It should be pointed out that COX-2 protein expression was strong in only 3 of 12 samples, weak in 2 of 12, and negative in 7 of 12 as detected by immunoblotting. Clearly the signal for COX-2 is weaker and less frequent than that observed in gastrointestinal tumors (2). To this end, it is not surprising that two reports have failed to detect COX-2 expression in a very limited number of ovarian cancer specimens (33, 34). Because COX-1 expression was reported to be expressed in ovarian cancer specimens by these two groups (33, 34), an explanation for these apparently discrepant data may depend on different relative amounts of COX-1 *versus* COX-2 gene products in ovarian cancer tissues due to sensitivity issues related to antibodies and methods of detection and/or because of different COX-enzyme expression patterns in different histological types of ovarian cancer. Indeed, we have published that very low COX-2 mRNA levels were detected in mucinous ovarian cancers when compared with gastric cancer specimens (35). In addition, our unpublished results indicate that frequency of COX-2 protein expression is relatively low in cancer cells derived from the mucinous type of tumors as detected by immunohistochemistry. All this indicates that the relative amount of COX-1 may be higher than that of COX-2 in ovarian carcinoma and that gastrointestinal tumors may express higher levels of COX-2 than ovarian tumors. However, thus far only expression of COX-2 but not that of COX-1 has been shown to associate with prognosis in ovarian cancer patients, indicating that assessing the expression levels of COX-2 protein is biologically meaningful.

The most frequent genetic alteration known in serous ovarian cancer is related to mutation of p53 tumor suppressor gene (12–14). The tubal (normal serous epithelium of müllerian origin) staining pattern was regarded in our work as a reference of normal (wild-type) p53 expression. In carcinomas, two categories distinct from the normal expression were observed: excessive p53 (overexpression) and completely negative p53. Interestingly, completely negative p53 conferred as poor prognosis as excessive p53 when compared with tumors showing normal p53 expression (44). Traditionally, p53 overexpression by immunohistochemistry is thought to represent *TP53* mutation, and p53 negative tumors are considered to carry wild-type *TP53.* However, high concordance of increased p53 protein is only seen with missense mutations, which result in protein that is resistant to degradation and has longer half-life than the wild-type counterpart. Other types of mutations (nonsense, insertion, deletion, and splice site aberrations) may result in truncated proteins that are functionally null but do not usually increase p53 protein stability. In addition to null mutations, the lost p53 immunostaining may be because of, for example, homozygous deletion or epigenetic silencing of the gene. In accordance with our result, one study reported the worst prognosis in stage III–IV ovarian carcinomas that showed null mutations of *TP53* and lacking p53 immunostaining (36). Furthermore, our present data imply that p53 negative and p53 overexpressing tumors contain more often high expression of COX-2 than the normal low pattern of p53 expression. In addition to ovarian cancer, which has been found in our work and those of others (31), association of COX-2 expression and p53 immunostaining has previously been found in gastric and breast cancers (37, 38). Because the *COX-2* gene has been shown to be induced in p53 defective cells and down-regulated by wild-type p53 (39, 40), there may exist a direct link between a defective p53 pathway and elevated levels of COX-2 expression in cancer cells.

In addition to p53, altered expression of another tumor

suppressor gene, SMAD4, has been detected in 28% of serous ovarian carcinomas (17). We found a positive correlation between elevated COX-2 expression and defective SMAD4 expression in the serous ovarian cancer specimens. It is not known whether SMAD4 can directly affect COX-2 expression. However, because tumor progression depends on cumulative genetic alterations, it is interesting to note that the frequency of COX-2 expression was higher in tumors with combined defect of p53 and SMAD4 when compared with the tumors with only one of the tumor suppressor genes affected. Elevated COX-2 expression was not restricted to p53 and SMAD4 aberrant tumors, and thus, other mechanisms are likely to be responsible for elevated COX-2 expression as well. In addition to tumor suppressor genes, function of several oncogenes is modified in ovarian cancer, which includes amplification and overexpression of HER-2/neu (18, 19). Overexpression of HER-2/neu induces COX-2 expression in mammary epithelial cells, and amplification of *HER-2/neu* correlated with COX-2 expression in breast cancer specimens (38, 41), but such association was not found in ovarian cancer as detected by immunohistochemistry (29). Our results show that elevated COX-2 expression in serous ovarian carcinoma correlates with amplification of *HER-2/neu* as detected by chromogenic *in situ* hybridization. It is interesting to note that combination of COX-2-selective drugs with inhibitors of HER-2/neu have provided an additive antitumor effect in experimental animal models (42, 43).

In conclusion, our results suggest that elevated expression of COX-2 associates with poor differentiation and with reduced survival in serous ovarian carcinomas. Expression of COX-2 associated with altered expression of tumor suppressor genes p53 and SMAD4 and with amplification of *HER-2/neu* oncogene, which may in part be responsible for induction of COX-2 expression in ovarian cancer.

ACKNOWLEDGMENTS

We thank Gynel Arifdshan, Tuija Hallikainen, Elina Laitinen, and Päivi Peltokangas for excellent technical assistance.

REFERENCES

1. Dannenberg, A. J., Altorki, N. K., Boyle, J. O., Dang, C., Howe, L. R., Weksler, B. B., and Subbaramaiah, K. Cyclo-oxygenase 2: a pharmacological target for the prevention of cancer. Lancet Oncol., *2:* 544–551, 2001.

2. Gupta, R. A., and DuBois, R. N. Colorectal cancer prevention and treatment by inhibition of cyclooxygenase-2. Nat. Rev. Cancer, *1:* 11–21, 2001.

3. Van Rees, B. P., and Ristimäki, A. Cyclooxygenase-2 in carcinogenesis of the gastrointestinal tract. Scand. J. Gastroenterol., *36:* 897–903, 2001.

4. Oshima, M., Dinchuk, J. E., Kargman, S. L., Oshima, H., Hancock, B., Kwong, E., Trzaskos, J. M., Evans, J. F., and Taketo, M. M. Suppression of intestinal polyposis in Apc δ716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). Cell, *87:* 803–809, 1996.

5. Chulada, P. C., Thompson, M. B., Mahler, J. F., Doyle, C. M., Gaul, B. W., Lee, C., Tiano, H. F., Morham, S. G., Smithies, O., and Langenbach, R. Genetic disruption of Ptgs-1, as well as Ptgs-2, reduces intestinal tumorigenesis in Min mice. Cancer Res., *60:* 4705–4708, 2000.

6. Liu, C. H., Chang, S. H., Narko, K., Trifan, O. C., Wu, M. T., Smith, E., Haudenschild, C., Lane, T. F., and Hla, T. Overexpression of cyclooxygenase-2 is sufficient to induce tumorigenesis in transgenic mice. J. Biol. Chem., *276:* 18563–18569, 2001.

7. Steinbach, G., Lynch, P. M., Phillips, R. K., Wallace, M. H., Hawk, E., Gordon, G. B., Wakabayashi, N., Saunders, B., Shen, Y., Fujimura, T., Su, L. K., and Levin, B. The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. N. Engl. J. Med., *342:* 1946–1952, 2000.

8. Friedlander, M. L. Prognostic factors in ovarian cancer. Semin. Oncol., *25:* 305–314, 1998.

9. Heintz, A. P., Odicino, F., Maisonneuve, P., Beller, U., Benedet, J. L., Creasman, W. T., Ngan, H. Y., Sideri, M., and Pecorelli, S. Carcinoma of the ovary. J. Epidemiol. Biostat., *6:* 107–138, 2001.

10. Pejovic, T., Himmelmann, A., Heim, S., Mandahl, N., Floderus, U. M., Furgyik, S., Elmfors, B., Helm, G., Willen, H., and Mitelman, F. Prognostic impact of chromosome aberrations in ovarian cancer. Br. J. Cancer, *65:* 282–286, 1992.

11. Taetle, R., Aickin, M., Panda, L., Emerson, J., Roe, D., Thompson, F., Davis, J., Trent, J., and Alberts, D. Chromosome abnormalities in ovarian adenocarcinoma: II. Prognostic impact of nonrandom chromosome abnormalities in 244 cases. Gene Chromosome Cancer, *25:* 46–52, 1999.

12. Marks, J. R., Davidoff, A. M., Kerns, B. J., Humphrey, P. A., Pence, J. C., Dodge, R. K., Clarke-Pearson, D. L., Iglehart, J. D., Bast, R., and Berchuck, A. Overexpression and mutation of p53 in epithelial ovarian cancer. Cancer Res., *51:* 2979–2984, 1991.

13. Hartmann, L. C., Podratz, K. C., Keeney, G. L., Kamel, N. A., Edmonson, J. H., Grill, J. P., Su, J. Q., Katzmann, J. A., and Roche, P. C. Prognostic significance of p53 immunostaining in epithelial ovarian cancer. J. Clin. Oncol., *12:* 64–69, 1994.

14. Klemi, P. J., Pylkkänen, L., Kiilholma, P., Kurvinen, K., and Joensuu, H. p53 protein detected by immunohistochemistry as a prognostic factor in patients with epithelial ovarian carcinoma. Cancer (Phila.), *76:* 1201–1208, 1995.

15. Arnold, N., Hägele, L., Walz, L., Schempp, W., Pfisterer, J., Bauknecht, T., and Kiechle, M. Overrepresentation of 3q and 8q material and loss of 18q material are recurrent findings in advanced human ovarian cancer. Gene Chromosome Cancer (Phila.), *16:* 46–54, 1996.

16. Tapper, J., Sarantaus, L., Vahteristo, P., Nevanlinna, H., Hemmer, S., Seppälä, M., Knuutila, S., and Butzow, R. Genetic changes in inherited and sporadic ovarian carcinomas by comparative genomic hybridization: extensive similarity except for a difference at chromosome 2q24–q32. Cancer Res., *58:* 2715–2719, 1998.

17. Lassus, H., Salovaara, R., Aaltonen, L. A., and Butzow, R. Allelic analysis of serous ovarian carcinoma reveals two putative tumor suppressor loci at 18q22–q23 distal to SMAD4, SMAD2, and DCC. Am. J. Pathol., *159:* 35–42, 2001.

18. Berchuck, A., Kamel, A., Whitaker, R., Kerns, B., Olt, G., Kinney, R., Soper, J. T., Dodge, R., Clarke-Pearson, D. L., and Marks, P. Overexpression of HER-2/neu is associated with poor survival in advanced epithelial ovarian cancer. Cancer Res., *50:* 4087–4091, 1990.

19. Meden, H., Marx, D., Roegglen, T., Schauer, A., and Kuhn, W. Overexpression of the oncogene c-erbB-2 (HER2/neu) and response to chemotherapy in patients with ovarian cancer. Int. J. Gynecol. Pathol., *17:* 61–65, 1998.

20. Klimp, A. H., Hollema, H., Kempinga, C., van der Zee, A. G., de Vries, E. G., and Daemen, T. Expression of cyclooxygenase-2 and inducible nitric oxide synthase in human ovarian tumors and tumor-associated macrophages. Cancer Res., *61:* 7305–7309, 2001.

21. Matsumoto, Y., Ishiko, O., Deguchi, M., Nakagawa, E., and Ogita, S. Cyclooxygenase-2 expression in normal ovaries and epithelial ovarian neoplasms. Int. J. Mol. Med., *8:* 31–3622, 2001.

22. Denkert, C., Köbel, M., Pest, S., Koch, I., Berger, S., Schwabe, M., Siegert, A., Reles, A., Klosterhalfen, B., and Hauptmann, S. Expression of cyclooxygenase 2 is an independent prognostic factor in human ovarian carcinoma. Am. J. Pathol., *160:* 893–903, 2002.

23. Kononen, J., Bubendorf, L., Kallioniemi, A., Bärlund, M., Schraml, P., Leighton, S., Torhorst, J., Mihatsch, M. J., Sauter, G., and Kallioniemi, O. P. Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat. Med., *4:* 844–847, 1998.

24. Ristimäki, A., Nieminen, O., Saukkonen, K., Hotakainen, K., Nordling, S., and Haglund, C. Expression of cyclooxygenase-2 in human transitional cell carcinoma of the urinary bladder. Am. J. Pathol., *158:* 849–853, 2001.

25. Saukkonen, K., Nieminen, O., van Rees, B., Vilkki, S., Härkönen, M., Juhola, M., Mecklin, J. P., Sipponen, P., and Ristimäki, A. Expression of cyclooxygenase-2 in dysplasia of the stomach and in intestinal-type gastric adenocarcinoma. Clin. Cancer Res., *7:* 1923–1931, 2001.

26. Tanner, M., Järvinen, P., and Isola, J. Amplification of HER-2/neu and topoisomerase IIα in primary and metastatic breast cancer. Cancer Res., *61:* 5345–5348, 2001.

27. Ristimäki, A., Garfinkel, S., Wessendorf, J., Maciag, T., and Hla, T. Induction of cyclooxygenase-2 by interleukin-1 α. Evidence for post-transcriptional regulation. J. Biol. Chem., *269:* 11769–11775, 1994.

28. Ferrandina, G., Lauriola, L., Zannoni, G. F., Fagotti, A., Fanfani, F., Legge, F., Maggiano, N., Gessi, M., Mancuso, S., Ranelletti, F. O., and Scambia, G. Increased cyclooxygenase-2 (Cox-2) expression is associated with chemotherapy resistance and outcome in ovarian cancer patients. Ann. Oncol., *13:* 1205–1211, 2002.

29. Ferrandina, G., Ranelletti, F. O., Lauriola, L., Fanfani, F., Legge, F., Mottolese, M., Nicotra, M. R., Natali, P. G., Zakut, V. H., and Scambia, G. Cyclooxygenase-2 (Cox-2), epidermal growth factor receptor (EGFR), and Her-2/neu expression in ovarian cancer. Gynecol. Oncol., *85:* 305–310, 2002.

30. Landen, C. N., Jr., Mathur, S. P., Richardson, M. S., and Creasman, W. T. Expression of cyclooxygenase-2 in cervical, endometrial, and ovarian malignancies. Am. J. Obstet. Gynecol., *188:* 1174–1176, 2003.

31. Shigemasa, K., Tian, X., Gu, L., Shiroyama, Y., Nagai, N., and Ohama, K. Expression of cyclooxygenase-2 and its relationship to p53 accumulation in ovarian adenocarcinomas. Int. J. Oncol., *22:* 99–105, 2003.

32. Buskens, C. J., van Rees, B. P., Sivula, A., Reitsma, J. B., Haglund, C., Bosma, P. J., Offerhaus, G. J., van Lanschot, J. J., and Ristimäki, A. Prognostic significance of elevated cyclooxygenase 2 expression in patients with adenocarcinoma of the esophagus. Gastroenterology, *122:* 1800–1807, 2002.

33. Dore, M., Cote, L. C., Mitchell, A., and Sirois, J. Expression of prostaglandin G/H synthase type 1, but not type 2, in human ovarian adenocarcinomas. J. Histochem. Cytochem., *46:* 77–84, 1998.

34. Gupta, R. A., Tejada, L. V., Tong, B. J., Das, S. K., Morrow, J. D., Dey, S. K., and DuBois, R. N. Cyclooxygenase-1 is overexpressed and promotes angiogenic growth factor production in ovarian cancer. Cancer Res., *63:* 906–911, 2003.

35. Ristimäki, A., Honkanen, N., Jänkälä, H., Sipponen, P., and Härkönen, M. Expression of cyclooxygenase-2 in human gastric carcinoma. Cancer Res., *57:* 1276–1280, 1997.

36. Shahin, M. S., Hughes, J. H., Sood, A. K., and Buller, R. E. The prognostic significance of p53 tumor suppressor gene alterations in ovarian carcinoma. Cancer (Phila.), *89:* 2006–2017, 2000.

37. Leung, W. K., To, K. F., Ng, Y. P., Lee, T. L., Lau, J. Y., Chan, F. K., Ng, E. K., Chung, S. C., and Sung, J. J. Association between cyclo-oxygenase-2 overexpression and missense p53 mutations in gastric cancer. Br. J. Cancer, *84:* 335–339, 2001.

38. Ristimäki, A., Sivula, A., Lundin, J., Lundin, M., Salminen, T., Haglund, C., Joensuu, H., and Isola, J. Prognostic significance of elevated cyclooxygenase-2 expression in breast cancer. Cancer Res., *62:* 632–635, 2002.

39. Subbaramaiah, K., Altorki, N., Chung, W. J., Mestre, J. R., Sampat, A., and Dannenberg, A. J. Inhibition of cyclooxygenase-2 gene expression by p53. J. Biol. Chem., *274:* 10911–10915, 1999.

40. Gallo, O., Schiavone, N., Papucci, L., Sardi, I., Magnelli, L., Frachi, A., Masini, E., and Capaccioli, S. Down-regulation of nitric oxide synthase-2 and cyclooxygenase-2 pathways by p53 in squamous cell carcinoma. Am. J. Pathol., *163:* 723–732, 2003.

41. Subbaramaiah, K., Norton, L., Gerald, W., and Dannenberg, A. J. Cyclooxygenase-2 is overexpressed in HER-2/neu-positive breast cancer. Evidence for involvement of AP-1 and PEA3. J. Biol. Chem., *277:* 18649–18657, 2002.

42. Howe, L. R., Subbaramaiah, K., Patel, J., Masferrer, J. L., Deora, A., Hudis, C., Thaler, H. T., Muller, W. J., Du, B., Brown, A. M., and Dannenberg, A. J. Celecoxib, a selective cyclooxygenase 2 inhibitor, protects against human epidermal growth factor receptor 2 (HER-2)/neu-induced breast cancer. Cancer Res., *62:* 5405–5407, 2002.

43. Mann, M., Sheng, H., Shao, J., Williams, C. S., Pisacane, P. I., Sliwkowski, M. X., and DuBois R. N. Targeting cyclooxygenase 2 and HER-2/neu pathways inhibits colorectal carcinoma growth. Gastroenterology, *120:* 1713–1719, 2001.

44. Lassus, H., Leminen, A., Lundin, J., Lehtovirta, P., and Butzow, R. Distinct subtypes of serous ovarian carcinoma identified by p53 determination. Gynecol. Oncol., *91:* 504–512, 2003.