

Cyclooxygenase-2 mediated regulation of E-cadherin occurs in conventional but not early-onset gastric cancer cell lines

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Abstract. *Background:* COX-2 and E-cadherin, involved in invasion and metastasis, are molecules critical for gastric carcinogenesis. A relationship between them is documented in non-small cell lung and prostate cancer. We present novel evidence of a relationship between COX-2 and E-cadherin expression in gastric cancer.

Methods: Using qPCR and Western blots analysis on celecoxib and PGE₂ treated and untreated gastric cancer cell lines derived from tumours of the intestinal type (MKN45, MKN28, AGS3, MKN7) and immunohistochemistry of 178 gastric cancers on tissue microarrays (TMA), we examined the COX-2/E-cadherin relationship.

Results: Down-regulation of COX-2 by celecoxib led to up-regulation of E-cadherin mRNA and protein levels in conventional gastric cancer cell lines, whereas expression was down regulated in the early-onset gastric cancer (EOGC) cell line. Immunohistochemistry on TMAs of 178 gastric cancers showed no correlation between COX-2 and E-cadherin expression in the conventional or early gastric cancer groups.

Conclusion: The results suggest that COX-2 has an impact on transcriptional regulation of E-cadherin in gastric cancer and our findings further highlight the intriguing nature of EOGCs which appear to have a molecular phenotype distinct from conventional gastric cancer. In addition, our findings also suggest that reduction of COX-2 using nonsteroidal anti-inflammatory drugs in gastric cancer chemoprevention may only be relevant for older patients.

Keywords: COX-2, E-cadherin, early-onset gastric cancer, CDH1 mutation, celecoxib

1. Introduction

Gastric cancer is the fourth most common malignancy worldwide and is still the second cause of cancer-related deaths in the world [27]. Patients diagnosed with gastric cancer usually have an advanced-stage disease leading to a poor prognosis. While the overall 5-year survival rate of gastric cancer patients is about 20%, that of patients with distant metastases is less than 5% [4,27]. Gastric cancer is the result of a combination of environmental factors and an accumulation of specific genetic alterations, and is more com-

mon in the older population. According to the Laurén classification, gastric cancer can be divided into two main histological types, diffuse and intestinal [16].

Around 10% of patients with gastric cancer belong to the early-onset type of gastric cancer (presenting <45 years old) in which it is postulated that genetic factors may play a more important role than in conventional types of gastric cancer (presenting >45 years old) [10,21].

COX-2 is an inducible isozyme of cyclooxygenase and produces prostaglandin E₂ (PGE₂) in response to various inflammatory stimuli or growth factors. Prostaglandin E₂ plays an important role in regulating diverse cellular functions under physiological and pathological conditions. Gastric adenocarcinomas express high levels of COX-2 when compared to non-neoplastic mucosa, where levels are low or unde-

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tectable [33,36]. COX-2 is predominantly expressed in intestinal-type gastric carcinomas and their precursor lesions and is overexpressed less commonly in the diffuse-type [20,35,40]. COX-2 overexpression has been associated with an inhibition of apoptosis [37], neoangiogenesis [39] and metastasis [24] and can be produced by stromal myofibroblasts [15]. We have found previously that COX-2 expression varies significantly between EOGCs and conventional cancers, with COX-2 overexpression occurring rarely in early-onset gastric cancers [20].

The transmembrane protein E-cadherin is known to play a crucial role during the progression of gastric cancer [23]. E-cadherin is necessary for maintaining normal epithelial tissue architecture and for stabilizing adherence junctions. It is more commonly dysregulated in diffuse gastric cancer [20] and germline mutations are found in hereditary diffuse gastric cancer [14]. Multiple mechanisms are responsible for the inactivation of E-cadherin in gastric cancer cells, such as gene mutation [1], promoter hypermethylation [19], post-translational truncation or modification [31], degradation by matrix metalloproteinase [18] and transcriptional repressors [6]. During carcinogenesis, the conversion of epithelial cells to fibroblastic phenotype occurs via a mechanism called epithelial-mesenchymal transition (EMT) and is often associated with down-regulation of E-cadherin. This reduction is an early stage in tumor invasion and metastasis in gastric cancer patients.

The first mention of a relationship between these proteins, which are so crucial in gastric cancer, was documented in non-small cell lung cancer where inhibition of tumor COX-2 using celecoxib led to increased E-cadherin expression [11]. Similarly, in prostate cancer the expression of E-cadherin and COX-2 were inversely correlated [30]. The elevation of COX-2 expression levels is an early event in gastric carcinogenesis [36] and is involved in invasion and metastasis, leading to the hypothesis that COX-2 may be a central element in gastric carcinogenesis. Non-steroidal anti-inflammatory drug (NSAID) users show a reduced risk of gastric cancer development in epidemiologic studies [13]. Celecoxib, a selective COX-2 inhibitor, is known to reduce the occurrence of polyps in the colon, and to prevent colorectal cancer [29,38]. However, the role of COX-2 appears to differ in the context of early-onset types of gastric cancer where little overexpression of COX-2 was found [20]. In light of this information, the relationship between COX-2 and E-cadherin expression in gastric cancer is of great interest.

The aim of this study was to investigate the correlation between COX-2 and E-cadherin expression pattern in gastric cancer, and due to the interesting pattern of COX-2 expression obtained previously using the immunohistochemical staining [20], we examined both early-onset and conventional gastric cancers types.

2. Materials and methods

2.1. Patients

A total of 85 conventional gastric cancers (patients >45 years old, diagnosed between 1993 and 2003), were obtained from the Academic Medical Centre, Amsterdam, The Netherlands. A total of 93 early-onset gastric carcinomas (patients <45 years old, 90% diagnosed between 1994 and 2002 and 10% diagnosed between 1980 and 1994), were obtained from 24 different institutions throughout the Netherlands through the nationwide database system, and from the Department of Pathology at the Jorvi Hospital (Espoo, Finland). The tumors were classified by an experienced gastrointestinal pathologist (GJAO) according to the Laurén classification as intestinal, diffuse or mixed gastric adenocarcinomas. Tissue microarrays were constructed of these two patient groups as described previously [20].

2.2. Immunohistochemistry

Sections (4 μ M) were deparaffinized and antigen retrieval was carried out by boiling for 10 min in 10 mM Tris/1 mM EDTA (pH 9). No antigen retrieval was used. Subsequently slides were immersed in 0.3% hydrogen peroxide in methanol for 30 min and nonspecific binding was blocked with 5% normal goat serum for 1 h at room temperature. The sections were incubated for 1 h at room temperature with the primary antibody, monoclonal mouse anti-E-cadherin (HECD-1) 1:100 dilution Abcam (Cambridge, UK). The Ultravision antipolyvalent HRP detection system (Lab Vision Corp., Fremont, CA, USA) was used to visualize antibody binding sites with 3,3'-diaminobenzidine as a chromogen. Sections were counterstained with hematoxylin. The specificity of various COX-2 antibodies has been extensively tested by our group, including the use of blocking peptides [35], in order to find the optimal antibody, which has subsequently been used in all COX-2 publications by our group. Immunohistochemistry for COX-2 was carried out as above with the following exceptions: antigen retrieval was carried

out in 0.01 M Na-citrate buffer (pH 6.0), followed by immersion in 0.6% hydrogen peroxide in methanol for 30 min and then in blocking solution (0.01 M Tris, 0.1 M MgCl₂, 0.5% Tween-20, 1% BSA, 5% normal goat serum) for 1 h. Incubation of the primary antibody was carried out using monoclonal COX-2 antibody at a dilution of 1:100 (Cayman Chemical Co., Ann Arbor, MI, USA) at 4°C overnight.

Scoring of immunohistochemistry was carried out for E-cadherin and COX-2 as follows. E-cadherin: 0 – no membranous staining, 1 – weak membranous staining (membranous staining in less than 10% of the cancer cells), 2 – membranous staining present in 10–50% of the cancer cells, 3 – >50% of the tumor cells stained with strong membranous staining. Categories 0 and 1 were designated E-cadherin-low; categories 2 and 3 were designated E-cadherin-high. As described previously [8], E-cadherin immunoreactivity in the cytoplasm was considered aberrant only if seen as discrete clumpy staining and accompanied by loss of membranous staining.

For COX-2 immunohistochemical staining, the following scoring criteria of the tumor cells were determined prior to analysis: 0 – no staining; 1 – weak dif-

fuse cytoplasmic staining (may contain stronger intensity in 10% of the cancer cells); 2 – moderate to strong granular cytoplasmic staining in 10–90% of the cancer cells; and 3 – more than 90% of the tumor cells stained with strong intensity. Scores 0 and 1 were categorized as COX-2 low and scores 2 and 3 as COX-2 high for the statistical analyses. A known COX-2 negative and positive tumor was used for each immunohistochemical assay, as positive and negative controls. Previously we have conducted a study whereby every 20th sample of the trial series was a known colon adenocarcinoma specimen, in which stromal cells at an area of ulceration were scored 3+, cancer cells from 2+ to 3+, and adjacent non-neoplastic epithelium 1+ [5]. This procedure confirmed that there was no significant intra-assay and interassay variability of the staining intensity and helped us score the trial specimens. All sections were reviewed by 2 of the authors independently. An example of the COX-2 scoring can be seen in Fig. 1.

Of note, for both COX-2 and E-cadherin, the scoring of the mixed carcinomas (10% of the total number of cancers) was done in exactly the same manner as with diffuse and intestinal tumors, i.e. by looking at the entire amount of tumor present (regardless of whether

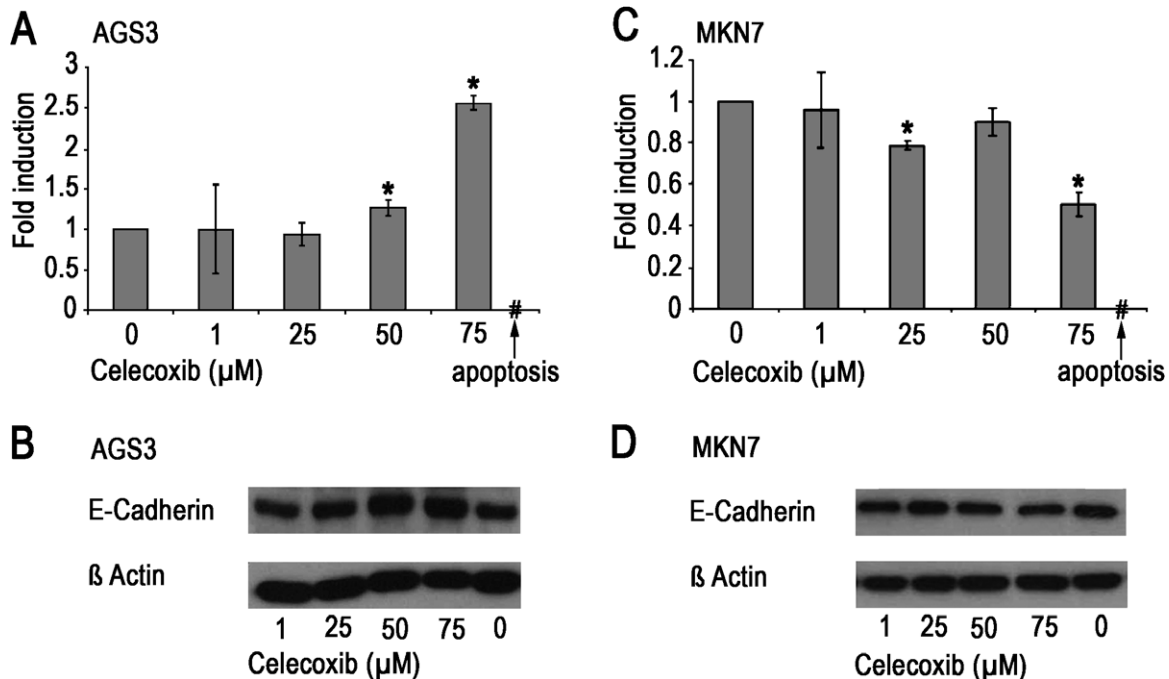


Fig. 1. The effect of celecoxib treatment in AGS3 and MKN7 cell lines. Cell lines were incubated with the indicated concentration of celecoxib for 24 h, and changes of E-cadherin mRNA and protein levels were determined as described in Section 2. (* $p < 0.05$ with respect to untreated cells using *t*-test.) AGS3 cell line (conventional gastric cancer), (A) qPCR and (B) Western blot results show fold increase in E-cadherin levels compared with untreated control. MKN7 cell line (EOGC), (C) qPCR analysis shows a dose-dependent decrease of E-cadherin mRNA whereas, (D) Western blot analysis showed a decrease in E-cadherin expression using software analysis, compared to untreated control.

this was diffuse or intestinal) and applying the scoring criteria as explained above.

2.3. Cell culture

Human gastric cancer cell lines, MKN45 (poorly differentiated (medullary) adenocarcinoma, age 62), MKN28 (intestinal adenocarcinoma (tubular), age 70) and AGS3 (intestinal adenocarcinoma, age 54) derived from conventional gastric cancer cell lines and MKN7 (well differentiated (tubular) adenocarcinoma, age 39) which belongs to the early-onset gastric cancer category) were cultured in RPMI-1640 (GIBCO BRL, MD, USA) supplemented with 10% fetal calf serum (GIBCO BRL) and 1% penicillin–streptomycin (GIBCO BRL) and maintained at 37°C at 5% CO₂ in air, as described previously [7].

2.4. Treatments and reagents

Cells were grown to approximately 60% confluence and were treated with increasing concentrations of celecoxib (ChemPacific, Baltimore, MD, USA) dissolved in DMSO (1 µM/l, 25 µM/l, 50 µM/l, 75 µM/l, 100 µM/l) and PGE-2 (Biomol International, L.P., Plymouth Meeting, PA, USA) diluted in ethanol (2 µg/ml, 4 µg/ml, 6 µg/ml, 8 µg/ml, 10 µg/ml) for 24 h at 37°C.

2.5. DNA isolation, purification and mutation analysis

DNA was extracted from gastric cancer cell lines using the QIAamp DNA Mini Kit (Qiagen, Venlo,

The Netherlands) according to the manufacturer's instructions. The DNA concentration used for each reaction was 50 ng, measured using the NanoDrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

Polymerase chain reaction (PCR) was performed in a 20 µl reaction containing 2.5 mM MgCl₂, 0.25 mM dNTPs, 0.5 µM of each primer, 0.5 U AmpliTaq Gold polymerase (Roche). The exons were amplified using the primers as described previously [2] with new primers designed for exon 1 as follows (forward) 5'-GTGAACCCTCAGCCAATCAG-3' and (reverse) 5'-AATGCGTCCCTCGCAAGT-3'. Amplification was performed with an initial denaturation step of 10 min at 95°C followed by 40 cycles of 95°C 30 s, 57–62°C (see Table 1) 1 min, 72°C 1.30 min, and then a final elongation step of 10 min at 72°C. PCR products were enzymatically purified by incubating at 37°C for 30 min with 5-U shrimp alkaline phosphatase (SAP, GE Healthcare) and 2-U Exonuclease I (Exo1, Westburg) followed by a 20 min incubation at 80°C to inactivate the enzymes. Samples were then subjected to direct sequencing using the BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems) and the ABI PRISM 3130xl (Applied Biosystems) Genetic Analyzer. The sequences were compared to reference sequence NT_010498 using Codon-Code Aligner software. Each mutation/variation was confirmed by a second run of PCR amplification and sequencing.

Table 1
Effect of mutation analysis on COX-2 regulation of E-cadherin in gastric cancer cell lines

Cell line	Type of gastric cancer	Exon	Mutation ¹	Protein changes ¹	Fold increase in E-cadherin expression after celecoxib at treatment (75 µM)	
					qPCR ²	Western blot ³
AGS3	Conventional gastric cancer	12	<i>c.1733-34insC</i>	<i>p.T578TfsX10</i>	2.56 ± 0.08 <i>p</i> = 0.004	2.66 ± 0.88
MKN45	Conventional gastric cancer	6/6	<i>c.823_832+8del18</i>	<i>p.A275_G278del</i>	2.72 ± 0.29 <i>p</i> = 0.004	1.32 ± 0.16
MKN28	Conventional gastric cancer	–	–	–	1.74 ± 0.17 <i>p</i> = 0.004	1.17 ± 0.10
MKN7	Early onset gastric cancer	–	–	–	0.5 ± 0.05 <i>p</i> = 0.003	0.74 ± 0.01

Notes: ¹ The results of mutation analysis are based on cDNA sequence in GenBank accession no. NM_004360.

²Data are the mean ± SD of three independent experiments performed in duplicate. In contrast to the control group, significance difference *p* < 0.05 (*t*-test).

³ Determination of protein expression levels of E-cadherin when cell lines were treated with 75 µM/l celecoxib. Data are the mean ± SD of at least two independent experiments, and are compared to the control group.

2.6. Western blot

Cells were lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 1% sodium dodecyl sulfate (SDS), 1 mM EDTA, 50 mM Tris pH 8.0) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na₃VO₄, 1 mM NaF and Complete mini protease inhibitor cocktail tablets (ROCHE Diagnostics GmbH, Mannheim, Germany), and then centrifuged at 14,000g for 20 min at 4°C. Proteins were separated on 10% SDS-PAGE and transferred to nitrocellulose membranes (Millipore Co., Bedford, MA, USA). Blots were blocked using 5% milk in PBST for 1 h at room temperature and then incubated overnight with the primary antibody (E-cadherin Abcam (1:100), COX-2 (1:250)) at 4°C followed by incubation with a horseradish-peroxidase-conjugated secondary antibody (1:7500) Biosource (Camarillo, CA, USA) for 1 h at RT. The antigen-antibody complex was detected using the enhanced chemiluminescence detection system (Amersham Biosciences, Buckinghamshire, UK) and exposed to Amersham HyperfilmTM ECL. Equal loading of samples was confirmed by probing the membranes with β -actin antibody (1:8000) (US Biological, MA, USA) (MP Biomedicals, LLC, Illkirch, France). For quantification of band intensities, Western blots were scanned with a high-resolution using an Opticom scanner (Isogen, De Meern, The Netherlands) and the relative intensities of protein bands were analyzed using TotalLab V 2003.03 software (Nonlinear Dynamics; Newcastle-upon-Tyne, UK). The results were replicated in at least two separate experiments.

2.7. RNA isolation, cDNA synthesis and quantitative PCR

RNA was extracted from cell lysate by homogenation in Trizol reagent (Invitrogen) followed by chloroform/phenol extraction. cDNA was prepared from 1 μ g of RNA using Reverse Transcriptase (Roche) and oligo-(dT) primers (Invitrogen). E-cadherin mRNA levels were quantified by quantitative PCR (qPCR) using the SYBR Green Quantitative PCR on an ABI7900 analyzer (Applied Biosystems, Warrington, UK). Amplification was carried out in a total volume of 20 μ l. PCR was performed with a first step of 10 min at 95°C followed by 40 cycles of 15 s at 95°C, 20 s at 60°C and 30 s at 72°C. Samples were run in duplicate and their relative expression was determined by normalizing the expression of each target to GAPDH. These were then compared to the normalized ex-

pression in a reference sample to calculate a fold change value. Primer sequences were as follows: human E-cadherin 5'-CGGGAATGCAGTTGAGGATC-3' and 5'-AGGATGGTGTAAAGCGATGGC-3', human GAPDH 5'-TGCACCACCAACTGCTTAGC-3' and 5'-GGCATGGACTGTGGTCATGAG-3'. Data were analysed using the SDS2.2.1 program (Applied Biosystems). Each experiment was performed at least three times in duplicate.

2.8. Statistical analysis

The SPSS 12 software package was used for statistical analysis. A Chi-squared test was used to determine whether the differences in expression levels found between antibodies were statistically significant ($p < 0.05$). A binary logistic regression model was used to adjust for potential confounding factors such as histological type. We used the t -test to show a difference between the expression of E-cadherin mRNA in celecoxib and PGE-2 treated cell lines compared to controls.

3. Results

3.1. Effect of celecoxib stimulation on gastric cancer cell lines

To investigate the effects of COX-2 regulation of E-cadherin, cell lines were treated with varying concentrations of celecoxib. All four cell lines exhibited inhibition of cell growth in a dose-dependent manner after celecoxib treatment. Celecoxib treatment for 24 h induced typical apoptotic morphological changes including cytoplasmic blebbing, aggregation and condensation of nuclear chromatin, and formation of apoptotic bodies.

All cell lines showed COX-2 and E-cadherin expression in normal cultured condition, prior to celecoxib treatment using Western blot analysis. Dose-dependent down-regulation of COX-2 by 75 μ M celecoxib was observed in AGS3, MKN45, MKN28 and MKN7 cell lines. The cell lines were subsequently examined for *E-cadherin* mRNA expression by qPCR. Dose-dependent up-regulation of *E-cadherin* mRNA by celecoxib at 75 μ M was found in AGS3, MKN45 and MKN28 conventional gastric cancer cell lines ($p = 0.004$, $p = 0.004$ and $p = 0.004$, respectively) compared to untreated controls, with a fold increase in *E-cadherin* mRNA of 2.56 ± 0.08 fold in AGS3,

2.72 ± 0.29 in MKN45 and 1.74 ± 0.17 in MKN28. Conversely, a significant down-regulation of *E-cadherin* mRNA of 0.5 ± 0.05 ($p = 0.003$) was seen after celecoxib incubation (75 µM) in MKN7, an early-onset gastric cancer cell line.

To explore whether the up-regulation or down-regulation of *E-cadherin* mRNA was accompanied by changes in the protein levels, Western blot analysis was performed. This analysis showed E-cadherin up-regulation with increasing celecoxib concentrations in AGS3, MKN45 and MKN28. Incubation with 75 µM concentration of celecoxib led to up-regulation of E-cadherin protein of 2.66 ± 0.88 fold in AGS3, 1.32 ± 0.16 fold in MKN45 and 1.17 ± 0.10 fold in MKN28, thus confirming that the increase in mRNA seen by qPCR, resulted in an actual increase in the E-cadherin protein. In the MKN7 cell line, expression of E-cadherin decreased by 0.74 ± 0.01, in line with the qPCR result.

Figure 1 shows the effect of celecoxib treatment in AGS3 and MKN7 cell lines.

3.2. Effect of PGE-2 stimulation on gastric cancer cell lines

The effect of PGE-2 exposure of 24 h on E-cadherin expression levels was studied in all four cell lines by qPCR and Western blot. No changes in E-cadherin mRNA or protein levels were seen in the AGS3, MKN45, MKN28 and MKN7 cell lines.

3.3. Relation between COX-2 and E-cadherin expression

The expression of E-cadherin and COX-2 was examined using a TMA of 85 conventional cancers and 93 EOGCs, as previously described [20]. COX-2 immunohistochemical staining was predominantly expressed in the cytoplasm. E-cadherin immunohistochemical expression was observed on the cell membrane at varying intensity with occasional clumpy cytoplasmic staining in the cytoplasm. Figure 2 is an example of E-cadherin and COX-2 immunohistochemical staining.

Using a Chi-squared test, a significant correlation between COX-2 and E-cadherin staining was seen in the EOGC group ($p = 0.005$). However, after using a binary logistic regression model to correct for histology (the EOGC is predominantly of the diffuse type) the correlation was no longer significant.

The immunohistochemical results can be seen in Table 2.

3.4. Mutation analysis of the E-cadherin gene in gastric cancer cell lines

We also investigated the genetic background of E-cadherin in the cell lines, in order to ascertain whether the presence of an E-cadherin mutation has any impact on the COX-2 regulation of E-cadherin. We tested AGS3, MKN45, MKN28 and MKN7 cell lines by analysing all 16 *E-cadherin* exons and exon-intron boundaries. The sequencing of the cell lines resulted in identification of two mutations that had occurred in the highly conserved sequence, coding for the E-cadherin extracellular domain. In AGS3 we found a single nucleotide insertion (C) in exon 12 after position 1733. The insertion leads to a premature termination at amino acid 588 resulting in a short form of E-cadherin (55KD) visible on Western blot instead of the normal length (120KD). In the MKN45 cell line, we confirmed the 18bp deletion in the region of the exon 6–intron 6 boundary starting from position –10 and ending at +8; the boundary was considered to be at position 0 [26]. This mutation leads to a four amino acid deletion at position 275–278. In AGS3 and MKN45 cell lines, only the mutant DNA sequence was seen, indicating that the wild type allele of *E-cadherin* was lost. No *E-cadherin* mutations were present in the genomic DNA of MKN28 and MKN7 cell lines. Interestingly, the cell lines with the strongest celecoxib upregulation of E-cadherin were also those containing E-cadherin mutations, as can be seen in Table 1.

4. Discussion

E-cadherin and COX-2 are involved in invasion and metastasis and have both been long known as critical molecules in the development of gastric cancer [23, 35,41]. The mention of a relationship between these proteins was documented more recently in non-small cell lung cancer [11] and prostate cancer [30] where loss of E-cadherin together with increased COX-2 expression was observed. A link between these two molecules has been only briefly examined in gastric cancer, where etodolac (COX-2 selective inhibitor) reduced cell proliferation and up-regulated the expression of *E-cadherin* mRNA in the gastric cancer cell line MKN45 [25].

Previously, we have shown that COX-2 expression is significantly different in EOGC compared to conventional gastric cancer, with the COX-2 high phenotype present in 66% of conventional gastric carcino-

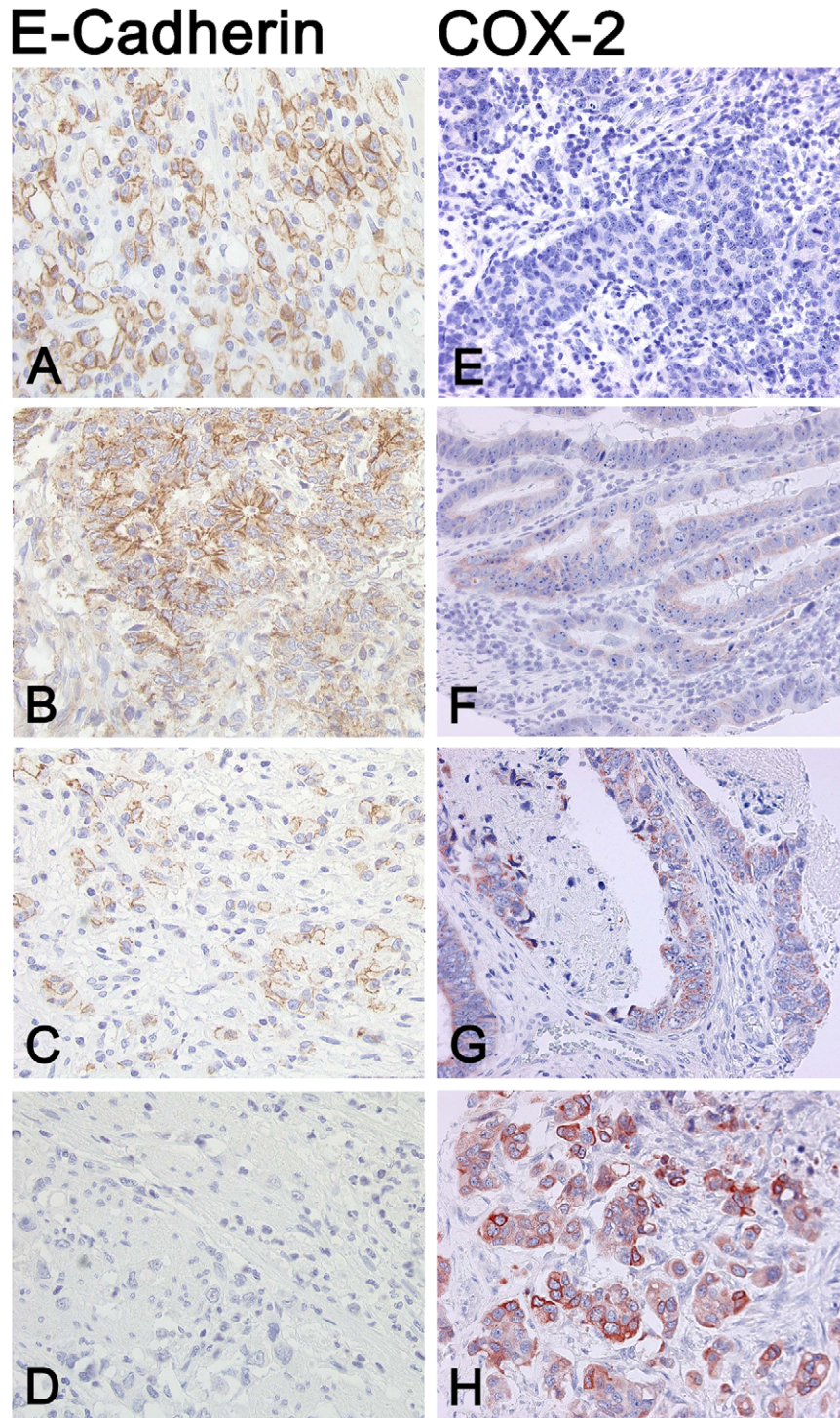


Fig. 2. Immunohistochemical staining for E-cadherin and for COX-2. E-cadherin staining: (A) category 3, >50% of the tumor cells stained with strong membranous staining, (B) category 2, moderate membranous staining in 10–50% of the cancer cells, (C) category 1, weak membranous staining (may contain membranous staining in less than 10% of the cancer cells or clumpy cytoplasmic staining), (D) category 0, no membranous staining. Categories 0 and 1 were E-cadherin-low; categories 2 and 3 were E-cadherin-high; COX-2 staining (E) category 0, no staining; (F) category 1, very weak diffuse cytoplasmic staining; (G) category 2, moderate-to-strong granular cytoplasmic staining in 10–50% and (H) category 3, more than 50% of tumor cells with strong intensity. Categories 0 and 1 were designated COX-2 low; categories 2 and 3 were designated COX-2 high.

Table 2
Results of immunohistochemistry

	COX-high	E-cadherin-low
Early-onset gastric cancer		
Diffuse (66)	1/66 (1.5%)	32/66 (48%)
Intestinal (20)	8/20 (40%)	7/20 (35%)
Mixed (7)	0 (0%)	4/7 (29%)
Conventional gastric cancer		
Diffuse (29)	16/29 (55%)	15/29 (52%)
Intestinal (47)	33/47 (70%)	7/47 (15%)
Mixed (9)	8/9 (89%)	4/9 (44%)

mas but in only 10% of early onset gastric cancers, and this remained significantly different when adjusted for histology [20]. Thus whether COX-2 regulation of E-cadherin in conventional gastric cancer occurs, and whether this differs in early-onset gastric cancer is of great interest.

Here we present the first report of a relationship between COX-2 and E-cadherin expression in different sub-types of gastric cancer. We find that down-regulation of COX-2 by celecoxib led to up-regulation of E-cadherin mRNA and protein levels in conventional gastric cancer cell lines.

In contrast to the dramatic effect seen in conventional cell lines, the MKN7 EOGC cell line showed a down-regulation of E-cadherin mRNA and protein levels after celecoxib treatment. This is not unexpected, considering the highly significant difference in expression of COX-2 seen between these two gastric cancer sub-types [20]. However, it is also of importance to consider, that an upregulation of E-cadherin *in vivo*, as would be suggested by the downregulation seen on COX-2 inhibition by celecoxib, would have no biological or evolutionary advantage to the tumour cells, and thus it probably represents an *in vitro* phenomenon only. In this study, all cell lines used were of the intestinal type. This reflects the difficulty of constructing diffuse cell lines [22] but in addition, it aids the comparisons between EOGC and conventional cell lines, as the differences in results seen are at least not due to different histological type. Inevitably, the extrapolating of this mechanism of COX-2 mediated E-cadherin regulation into primary tumours *in vivo*, at this point remains somewhat speculative, as are all findings which have been discovered through manipulation of cell lines.

Interestingly, this clear relationship between COX-2 and E-cadherin in gastric cancer cell lines was not

replicated by the TMA immunohistochemical findings, where after adjusting for histology, no correlation between COX-2 expression and E-cadherin expression was found. This may implicate a temporal relationship of this COX-2/E-cadherin interaction which cannot be seen in paraffin embedded material as it reflects only one time point of the cells. Additionally, it is known that COX-2 overexpression can be observed at the invasive front of gastric tumors [42], and it remains possible that COX-2 regulation of E-cadherin may be a local event occurring at distinct sites during tumor progression in gastric cancer, which would not be reflected in a TMA, where tumor has mainly been sampled from the middle of the tumor.

When considering a COX-2 dependent mechanism of E-cadherin regulation, the effect of an E-cadherin mutation on such a mechanism warrants investigation in order to ascertain whether they are mutually exclusive. Interestingly, in the two cell lines where a *CDH1* mutation was found, a significant up-regulation of E-cadherin occurred in response to celecoxib stimulation even in the presence of the mutations and was observed on both mRNA and protein levels. Methylation of the promoter region is also a commonly occurring mechanism in downregulation of E-cadherin in carcinogenesis, and it would be of interest to investigate whether COX-2 mediated E-cadherin regulation could still occur in the presence of E-cadherin promoter hypermethylation.

The mechanism by which COX-2 mediated E-cadherin regulation occurs, has been said to involve the transcriptional repressors ZEB1 and Snail in non-small cell lung cancer (NSCLC). Prostaglandin E2 has been shown to down-regulate E-cadherin expression in NSCLC by up-regulating ZEB1 and Snail [32]. The findings implicate PGE2 as an autocrine or paracrine modulator of ZEB1 and Snail and define a pathway by which COX-2 decreases E-cadherin expression in NSCLC by binding the E-boxes present in the E-cadherin promoter [11].

However, no significant association between Snail and SNAI2 and clinical parameters has been observed, and contradictory expression data for the same tumour type have been reported by different groups [3,9,12]. These discrepancies might be due to technical issues, possibly derived from the undefined specificity of most commercial anti-SNAI1 and 2 antibodies and, importantly, from the inappropriate assessment of nuclear staining and/or the discrimination between cytoplasmic and nuclear Snail stain found in most studies. Unfortunately, these drawbacks mean that the data avail-

able regarding Snail and SNAI2 expression in tumour samples must be interpreted with care [28]. In addition, although studies on the expression of Snail by reverse transcription PCR (RT-PCR) from whole tumour samples might overcome the uncertainties derived from immunohistochemistry, they are not suitable for differential cellular localization or detection in specific tumour regions such as invasive areas. Furthermore, additional problems with interpretation of RT-PCR data can arise from the existence of a *SNAIIP* human retrogene with a sequence very similar to *SNAI1* but whose expression is not correlated with invasive or metastatic behaviour [17]. We have examined both Snail and ZEB-1 using immunohistochemistry and Western blot analysis, yet the results obtained were neither reliable nor reproducible in our gastric cancer cohort of 178 patients (data not included). In addition, there are suggestions that SIP-1 acts as a repressor of E-cadherin in intestinal type gastric cancer, whereas Snail acts in diffuse type gastric cancer [34]. How these repressors may be involved in COX-2 mediated E-cadherin regulation in gastric cancer is yet to be accurately defined.

In summary, this is the first report to identify a relationship between COX-2 and E-cadherin expression in different sub-types of gastric cancer. The results show that COX-2 has an impact on transcriptional regulation of E-cadherin in gastric cancer and it is possible that inhibition of COX-2 function can help maintain the integrity of tumor cells, restore E-cadherin expression and prevent the progression of gastric cancer to distant metastases in patients with a conventional gastric cancer. An explanation for the disparate expression of COX-2 in different sub-types of gastric cancers and COX-2 driven regulation of E-cadherin remains of great interest. Our findings highlight the intriguing nature of EOGCs which appear to have a distinct molecular phenotype and suggest that reduction of COX-2 using nonsteroidal anti-inflammatory drugs in gastric cancer chemoprevention may only be relevant for older patients.

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