Cyclooxygenase-2/carbonic anhydrase-IX up-regulation promotes invasive potential and hypoxia survival in colorectal cancer cells

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Abstract

Inflammation promotes colorectal carcinogenesis. Tumour growth often generates a hypoxic environment in the inner tumour mass. We here report that, in colon cancer cells, the expression of the pro-inflammatory enzyme cyclooxygenase-2 (COX-2) associates with that of the hypoxia response gene carbonic anhydrase-IX (CA-IX). The COX-2 knockdown, achieved by the stable infection of a COX-2 specific short harpin RNA interference (shCOX-2), down-regulates CA-IX gene expression. In colorectal cancer (CRC) cells, PGE_2, the main COX-2 gene products, promotes CA-IX gene expression by ERK1/2 activation. In normoxic environment, shCOX-2 infected/CA-IX siRNA transfected CRC cells show a reduced level of active metalloproteinase-2 (MMP-2) that associates with a decreased extracellular matrix invasion capacity. In presence of hypoxia, COX-2 gene expression and PGE_2 production increase. The knockdown of COX-2/CA-IX blunts the survival capability of CRC cells in hypoxia. At a high cell density, a culture condition that creates a mild pericellular hypoxic environment, the expression of COX-2/CA-IX genes is increased and triggers the invasive potential of colon cancer cells. In human colon cancer tissues, COX-2/CA-IX protein expression levels, assessed by Western blot and immunohistochemistry, correlate each other and increase with tumour stage. In conclusion, these data indicate that COX-2/CA-IX interplay promotes the aggressive behaviour of CRC cells.

Keywords: COX-2 • inflammation • CA-IX • hypoxia • colon cancer

Introduction

Colorectal cancer (CRC) is a leading cause of cancer-related mortality in Western countries [1]. Clinical and experimental data show that inflammation promotes CRC in animal models and human beings [2–4]. Epidemiological reports indicate that the long-term administration of anti-inflammatory drugs decreases the risk of colon carcinoma, as well as induces the regression of polyp growth in familial adenomatous polyposis patients [5]. This phenomenon has been linked to the inhibition of cyclooxygenase-2 (COX-2), the enzyme that synthesizes the pro-inflammatory mediators PGE_2 [6]. COX-2 and PGE_2 levels are increased in colorectal carcinomas and promote invasiveness, angiogenesis and metastatic potential in CRC cells [7–10]. In animal models, *COX-2* gene knockout (KO), or COX-2 inhibition by selective drugs reduce tumour incidence, growth rate and tumour multiplicity [11]. COX-2 expression plays an important role in hypoxia survival of colon cancer cells [12]. Hypoxia occurs as a consequence of solid tumours growth and represents a key promoting regulatory factor for cancer growth and metastasis development [13–16]. Carbonic anhydrase-IX (CA-IX) is

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one of the genes that gained much attention as a possible marker for tumour hypoxia *in vivo* [17]. CA-IX protein expression also correlates with poor prognosis in solid tumours [18, 19]. Interestingly, CA-IX protein is expressed only in a fraction of CRC hypoxic cells, suggesting that micro-environmental factors, other than hypoxia, up-regulate *in vivo* CA-IX expression [20]. In fact, *CA-IX* gene expression is induced by hypoxia-independent mechanisms, such as the activation of ERK pathway [21–23]. Notably, ERK1/2 pathway activation is triggered by COX-2/PGE_2 axis up-regulation [24]. We here sought to investigate the interplay between *COX-2* and *CA-IX* genes in CRC cells and we explored the correlation between the expression of COX-2 and CA-IX proteins in CRC tissues.

Methods

Cell culture

Human colon cancer cells HT-29, HCT-116, HCA-7 and Caco-2 were obtained from American Type Culture Collection (Manassas, VA, USA), cultured at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Cambrex Bio Science, Bergamo, Italy) supplemented with 10% heat-inactivated foetal calf serum (FCS). Hypoxia culture condition (2% O_2) was generated in a humidified incubator supplied with 95% N_2/5% CO_2 (Thermoforma, Thermo, Waltham, MA, USA). Desferoxamine (DFX) and dimethylsulphoxide (DMSO) were purchased from Sigma (Sigma Chemical, St. Louis, MO, USA). The selective inhibitor SC-236 was purchased from Calbiochem (Calbiochem, San Diego, CA, USA). Cell death was evaluated by Trypan blue staining (Sigma): both floating and attached cells were assessed for cell death assay.

PGE_2 ELISA assay

PGE_2 levels were measured by ELISA (Cayman Chemical, Ann Arbor, MI, USA). HT-29 cells (5×10^5 cells/well) were seeded in a 6-well plate in 2 ml of fresh medium. After 48 hrs, supernatants were collected and centrifuged at $400 \times g$ for 5 min. at room temperature, then 100 μl of supernatant from each sample were used for the test. Samples were measured in triplicate and this experiment was repeated three separate times ($n = 3$).

COX-2 specific short hairpin RNA (shRNA)

Constructs coding for anti-COX-2 shRNA were prepared as described elsewhere [25]. Forward and reverse sequences for anti-COX-2 shRNA construct were 5′-GATCCCCAACTGCT CAACACCGGAATTTCAAGAG AATTCCGGTGTTGAGCAGTTTTTTTGGAA-3′ and 5′-AGCTTTTCCAAAAAAACTG CTCAACACCGGAATTCT CTTGAAATTCCGGTGTTGA GCAGTTGGG-3′, respectively, 64 nucleotides-containing oligos synthesized and purchased from Proligo (Proligo, Denver, CO, USA). Steps for cloning oligonucleotides into pSUPER.retro vector were made accordingly to pSUPER RNAi system protocol (www.oligoengine.com).

CA-IX and COX-2 specific short interfering RNA (siRNA)

CA-IX and appropriate control, scramble, SCR, siRNA were purchased from Invitrogen (Invitrogen, Carlsbad, CA, USA). COX-2/SCR siRNA were purchased from Proligo [25].

Luciferase assay and transfection procedure

Carbonic anhydrase promoter activity was assessed by using a pGL-3 vector containing a luciferase gene under the control of a −174/+63 fragment of the carbonic anhydrase IX promoter (CA−IX Luc, kindly provided by Dr. J. Pastorek, Slovak Academy of Sciences, Slovak Republic, Bratislava). Sixty percent confluent cells, plated on 0.75 cm^2 wells, were co-transfected with 500 ng of CA-IX Luc and 20 ng of Thymidine Kinase promoter driven Renilla Luciferase gene reporter vector (TK-Renilla, Promega, Madison, WI, USA) to control for transfection efficiency. All the transfection procedures were performed using Lipofectamine 2000. Luciferase activity was assessed by Dual-Luciferase® Reporter Assay System according the manufacturer's instructions (Promega, Madison, WI, USA).

Cell invasion assay and gelatin zymography

Invasion assay was performed using Boyden chambers with 8 μm pore polycarbonate membranes (New Technologies Group, Italy). Metalloproteinase-2 (MMP-2) activity was determined by gelatin zymography. Briefly, proteins of collected media were precipitated with 1:4 (vol/vol) ice-cold methanol overnight at −20°C, solubilized with sample buffer in absence of mercaptoethanol (1 M Tris-HCl pH 6.8, 2% SDS, 10% glycerol) and loaded into 10% SDS-polyacrylamide gel containing 1 mg/ml gelatine (Sigma). Gel was then incubated in a developing buffer (100 mM Tris-HCl, 10 mM $CaCl_2$, 20 mM NaCl pH 7.6) overnight at 37°C, stained for 2 hrs with 1% Coomassie Brilliant Blue R-250, and finally de-stained in a solution containing 10% acetic acid and 40% methanol. MMP-2 proteolytic activity was quantified using a semi-automated image analysis system (GelDoc, Biorad, Hercules, CA, USA).

Patients, immunohistochemistry (IHC) procedure and quantitative analysis

Samples from 87 sporadic CRC patients, histologically classified and graded according to WHO guidelines and pTNM (UICC) pathological staging criteria, were assessed in this investigation (Table 1) [26]. Formalin-fixed, paraffin-embedded specimens were available from 82 patients. Tissue samples frozen in liquid nitrogen, immediately after surgical procedure, were available from 17 patients, including 12 belonging to the above 82 cases set. For IHC, tissue sections were stained using polyclonal goat anti-COX-2 (C-20) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and monoclonal mouse anti-CA-IX (M-75, kindly provided by Dr. J. Pastorek) [27, 28]. COX-2 and CA-IX protein expression were evaluated using a semi-quantitative score, called Multiplicative Quickscore, MQ [29]. Briefly, the percentage of immuno-positive cells at each field (100 X) was scored as follows: Percentage ≤1% = Score 0, > 1% ≤25% = Score 1, >25% ≤50% = Score 2, >50% ≤75% = Score 3, >75% = Score 4; Staining intensity was scored as: Weak = Score 1, Moderate = Score 2,

Table 1 Clinico-pathological characteristics of CRC patients

Features	Patients $n = 87$	
	n	%
Age, years		
Mean	69.3 ± 1.4	
Range	32–89	
Sex		
Female	37	42.5
Male	50	57.5
Lesion site		
Right colon	35	40.2
Left colon	52	59.8
Tumour diameter (mm)		
Mean	46.3 ± 1.7	
Range	5.5–75	
Histological grading		
Poorly differentiated	8	9.2
Moderately differentiated	64	73.6
Well differentiated	15	17.2
Pathological staging		
Stage I	10	11.5
Stage II	35	40.2
Stage III	35	40.2
Stage IV	7	8.1

Strong = Score 3. The product of percentage and intensity mean values was grouped as follows: <1 = Negative (NEG), ≥1 <4 = LOW, ≥4 <8 = Intermediate (INT), ≥8 = HIGH. Tissues were obtained after patients written informed consent and the use of tissues was approved by the local ethics committee.

Protein extraction and Western blot analysis

Frozen tissues and cells were homogenized for 15 min. using lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$, 1% Triton X-100, 10% glycerol, 2 mM EGTA, 1 mM DTT) containing protease and phosphatase cocktail inhibitors (Sigma). Samples were processed according to standard procedures (see Table S1).

RNA extraction and RT-PCR analysis

Total RNA was isolated using TRIzol Reagent and reverse-transcribed using Superscript II (Invitrogen). Primers and annealing temperatures are described in Table S2. The PCR products were separated through 1.8–2.5% agarose gel.

Statistical analysis

Continuous variables were analysed by ANOVA test (unequal variance assumed) followed by *post hoc* test adjusted for multiple comparisons. Correlation between variables was assessed by Spearman correlation coefficient. Ordinal and non-normal distributed variables were analysed by non-parametric rank test (Mann–Whitney test). All the statistical analyses were performed using SPSS 10.1 Package (SPSS, Chicago, IL, USA).

Results

COX-2 expression and PGE_2 production promote an ERK-dependent CA-IX up-regulation

This investigation started with the observation that high COX-2 expression level is accompanied by the up-regulation of CA-IX protein in colorectal cell lines (Fig. 1A). We therefore sought to investigate the functional relationship between COX-2 and CA-IX gene expression. We purposely examined HT-29 cells sub-population infected with a pSUPER.retro vector, encoding a COX-2 specific (shCOX-2) or control (shCTR) short hairpin RNA. RT-PCR and Western blot (WB) analyses revealed that shCOX-2 HT-29 infected cells showed reduced levels of CA-IX mRNA and protein compared to shCTR infected cells (Fig. 1B). Given that CA-IX gene is regulated by two molecular pathways, namely HIF-1α [30] and MAP kinase ERK1/2 [21–23], we then evaluated the protein levels of HIF-1α, ERK1/2 and phosphorylated ERK1/2 (pERK) in shCTR/COX-2 HT-29 infected cells. We found lower amounts of pERK protein in shCOX-2 HT-29 infected cells compared to shCTR HT-29 infected ones, whereas no HIF-1α expression was found in such a culture condition (Fig. 1C). Moreover, due to the capability of COX-2 and PGE_2 activate ERK pathway, we tested the role of ERK1/2 protein in COX-2/CA-IX interplay. We found that the PGE_2-dependent up-regulation of CA-IX gene was halted by the administration of the MEK1/ERK inhibitor UO-126 (Fig. 1D). The PGE_2/ERK-dependent regulation of CA-IX gene expression was then evaluated by CA-IX gene promoter activity assay (CA-IX Luc) in HT-29 cells. We observed a lower level of CA-IX Luc activity in shCOX-2 HT-29 infected compared to shCTR infected cells, and we found that PGE_2 administration triggered an ERK-dependent increase of CA-IX Luc activity (Fig. 1E). Overall, these data indicate that COX-2 gene up-regulation, by means of PGE_2/ERK activation, promotes CA-IX gene expression in colon cancer cells.

COX-2/CA-IX interplay promotes invasiveness of colon cancer cells

ERK activation promotes the invasive behaviour of cancer cells [31, 32]. We therefore assessed the role of COX-2/CA-IX interplay

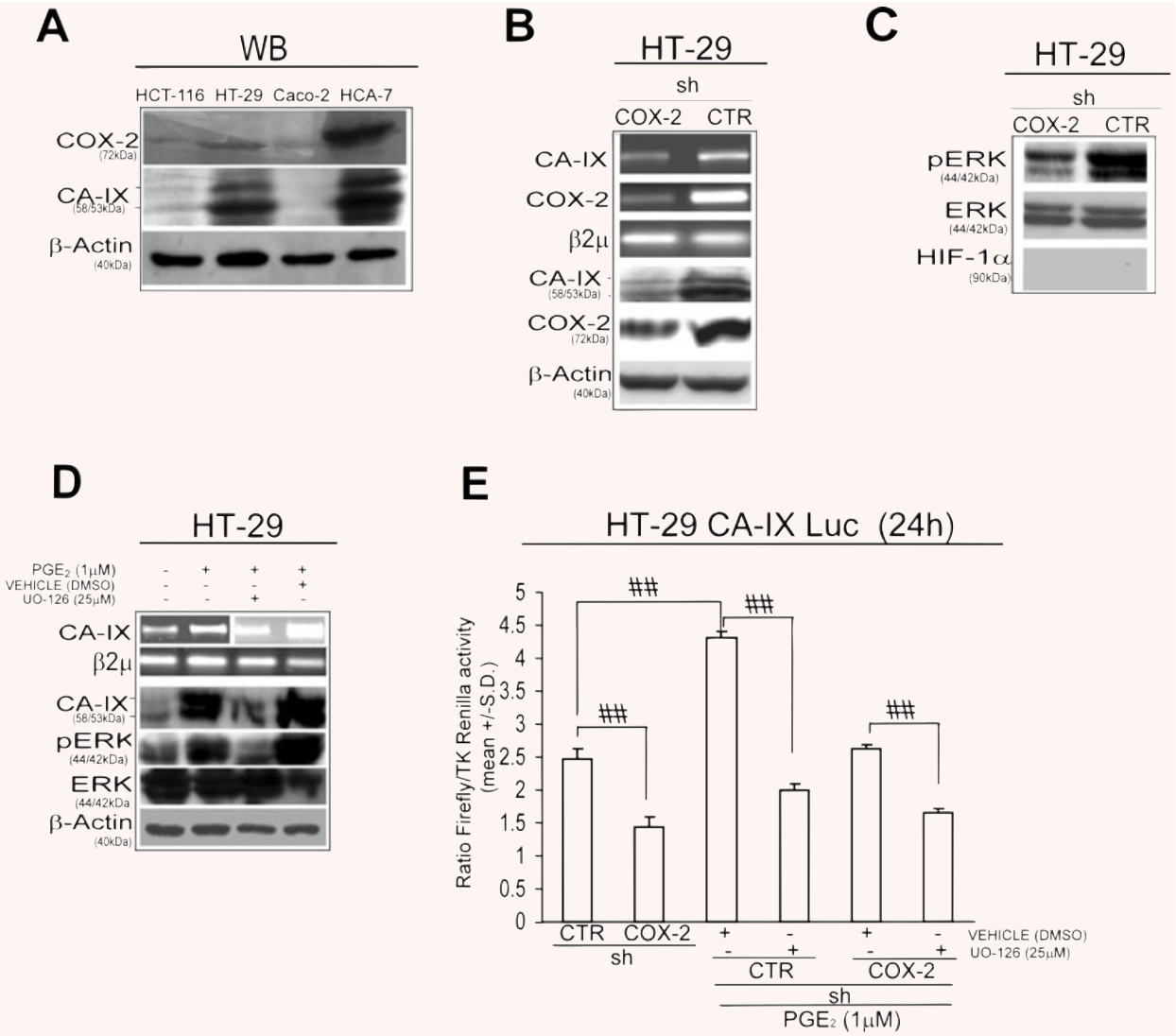


Fig. 1 COX-2 up-regulates CA-IX gene expression. (**A**) Western blot (WB) analysis of CA-IX and COX-2 protein level in 4 colon cancer cell lines: HCT-116, HT-29, Caco-2 and HCA-7. (**B**) HT-29 cells, stably infected with a pSUPER.retro vector encoding a COX-2 specific (shCOX-2) or control (shCTR) short hairpin RNA: RT-PCR analysis of CA-IX and COX-2 mRNA level, WB analysis of CA-IX, COX-2 and β-Actin. (**C**) WB analysis of phosphorylated ERK 1/2 (pERK), total ERK and HIF-1α protein level in shCTR/COX-2 HT-29 infected cells (β-Actin is reported in panel B). (**D**) HT-29 cells administered with PGE_2 (1 μM) for 24 hrs in presence/absence of UO-126 (25 μM, 1 hr pre-treatment) or control vehicle DMSO: RT-PCR analysis of CA-IX mRNA level and WB analysis of CA-IX, pERK and ERK protein level. (**E**) Luciferase assay of −174/+63 fragment of CA-IX promoter (CA-IX Luc) in shCTR/COX-2 HT-29 infected cells, in presence/absence of PGE_2 (1 μM) and/or UO-126 (25 μM) or DMSO for 24 hrs. Luciferase activity is expressed as a ratio over Thymidine Kinase (TK) driven Renilla Luciferase activity. Beta-2 microglobulin (β2μ) was assessed as quantitative control for RT-PCR analysis. β-Actin was assessed as quantitative control for WB analysis. Data are expressed as mean ± S.D. of three replicates ($n = 3$) (ANOVA test, $^{\#\#}P < 0.0001$, *post hoc* test corrected for multiple comparison).

in the regulation of the invasive potential of CRC cells. We found that shCOX-2 HT-29 infected cells and HT-29 cells transiently transfected with a CA-IX specific siRNA disclosed a reduced invasive potential compared to matched controls (Fig. 2A). Notably, no cell death was observed in such experimental conditions (see Fig. 2A).

Due to the strict connection between COX-2 and metalloproteinases (MMPs) expression and activity [33, 34], we hypothesized that CA-IX may control the activation of MMPs [35, 36]. Western blot analysis revealed lower levels of extracellular/active, but not intracellular/inactive, MMP-2 protein level in shCOX-2 HT-29 infected cells and in CA-IX siRNA HT-29 transfected cells

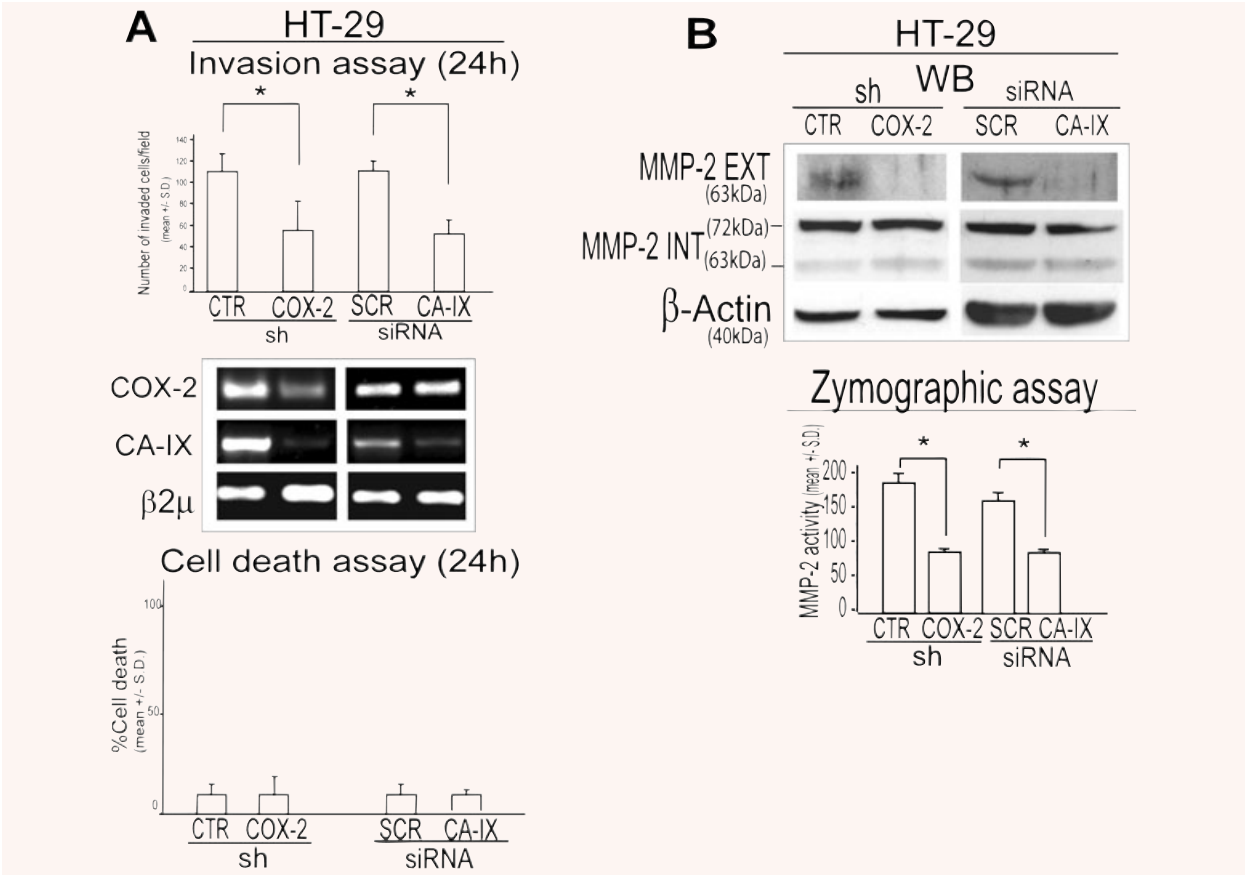
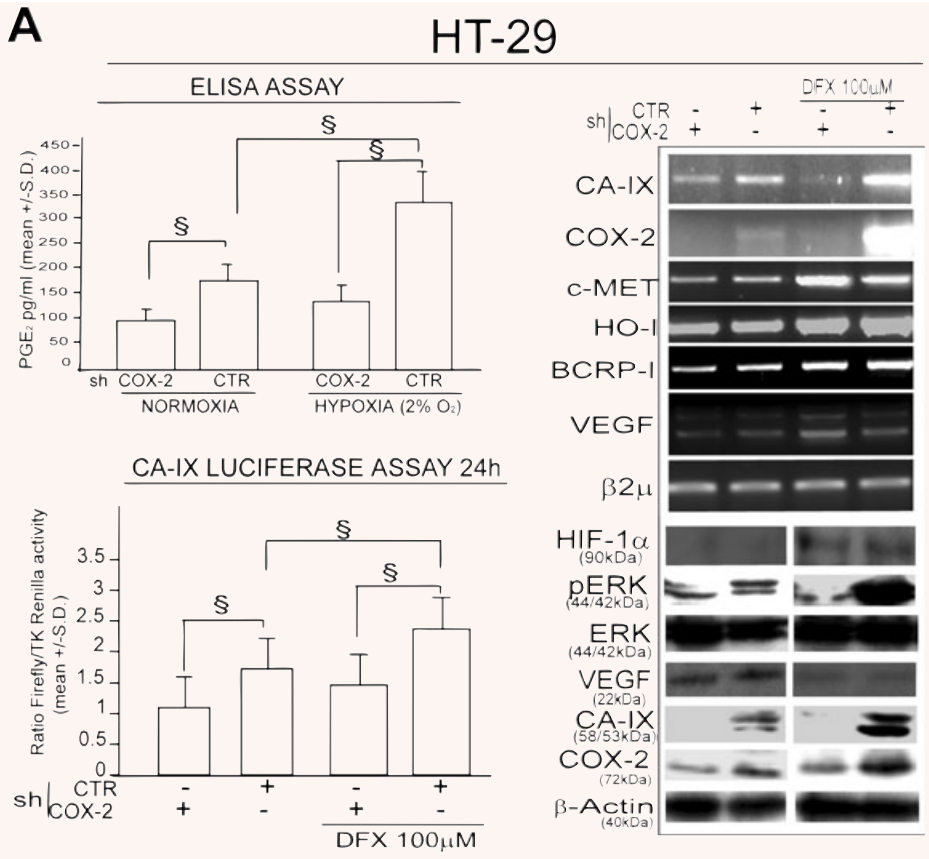


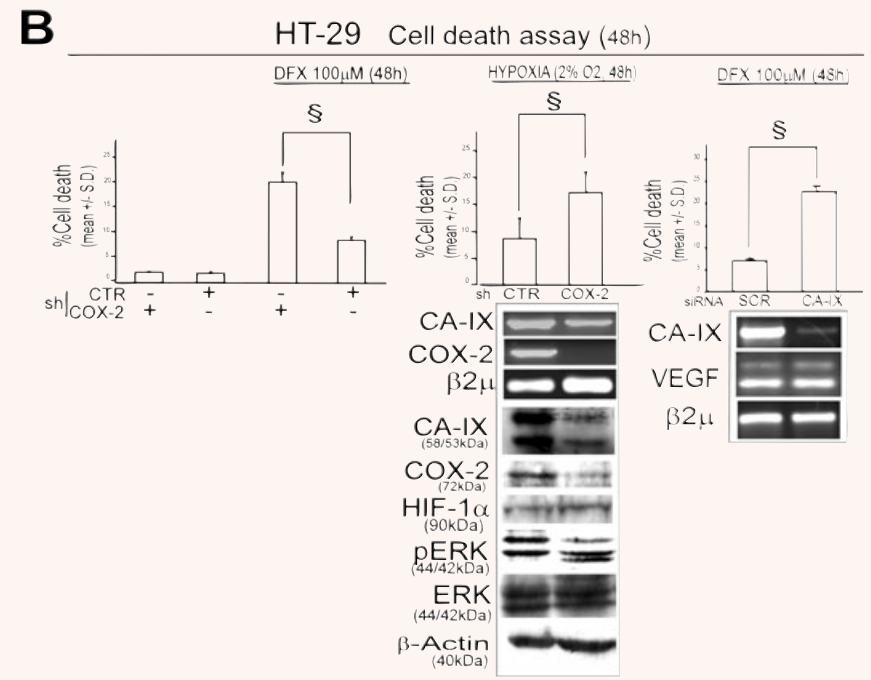
Fig. 2 COX-2/CA-IX interplay promotes invasive potential of colon cancer cells. (**A**) Boyden chamber invasion assay (24 hrs), RT-PCR analysis of COX-2 and CA-IX mRNA level and cell death assay (24 hrs) in shCTR/COX-2 HT-29 infected cells and in HT-29 cells transiently transfected with a CA-IX specific or scramble (SCR) siRNA (1 μg, 48 hrs pre-exposure). (**B**) WB analysis of active, extracellular (EXT) and inactive, intracellular (INT) protein levels and zymographic assay of MMP-2 in shCTR/COX-2 HT-29 infected cells and SCR/CA-IX siRNA transiently transfected HT-29 cells (1 μg, pre-exposure for 48 hrs). β2μ was assessed as quantitative control for RT-PCR analysis. β-Actin was assessed as quantitative control for WB analysis. Data are expressed as mean ± S.D. of three replicates ($n = 3$) (ANOVA test, $^*P = 0.001$).

compared to their matched controls (Fig. 2B). Moreover, zymographic assay showed a reduced activity of MMP-2 enzyme in shCOX-2 HT-29 infected cells and in CA-IX siRNA HT-29 transfected cells, compared to their matched controls (Fig. 2B). These data suggest that the COX-2/CA-IX interplay promotes the invasive capacity of colon cancer cells.

COX-2 gene expression enhances CA-IX-dependent hypoxia survival

CA-IX is a hypoxia survival gene [17, 23]. Recent data show that COX-2 is up-regulated upon hypoxia exposure [12]. We therefore sought to investigate the regulation of the COX-2/CA-IX pathway in presence of hypoxia, by exposing shCTR/COX-2 HT-29 infected cells to the hypoxia mimetic Desferoxamine (DFX, 100μM) and to hypoxic culture conditions (2% O_2) [12]. We found that PGE_2 production was increased upon 2% O_2 exposure as well as that CA-IX promoter activity and mRNA level was increased upon exposure to DFX 100 μM in shCTR, but not in shCOX-2 HT-29 infected cells (Fig. 3A). ShCOX-2 infected cells disclosed a higher susceptibility to DFX or 2% O_2 induced cell death, a phenomenon that was also observed in HT-29 cells transfected with CA-IX siRNA, compared to matched controls (Fig. 3B). PGE_2 administration yielded an increase in the survival capacity of HT-29 cells in presence of DFX 100μM that was hampered by the co-administration of UO-126 (Fig. 3C). The role of COX-2/CA-IX interplay in hypoxia survival of colon cancer cells was confirmed in HCT-116 and HCA-7 colon cancer cells in which we found that the exposure to DFX promotes the expression of CA-IX mRNA in a COX-2-dependent fashion (Fig. S1). Finally, the administration of the COX-2 specific inhibitor SC-236 down-regulated CA-IX mRNA level and triggered cell death





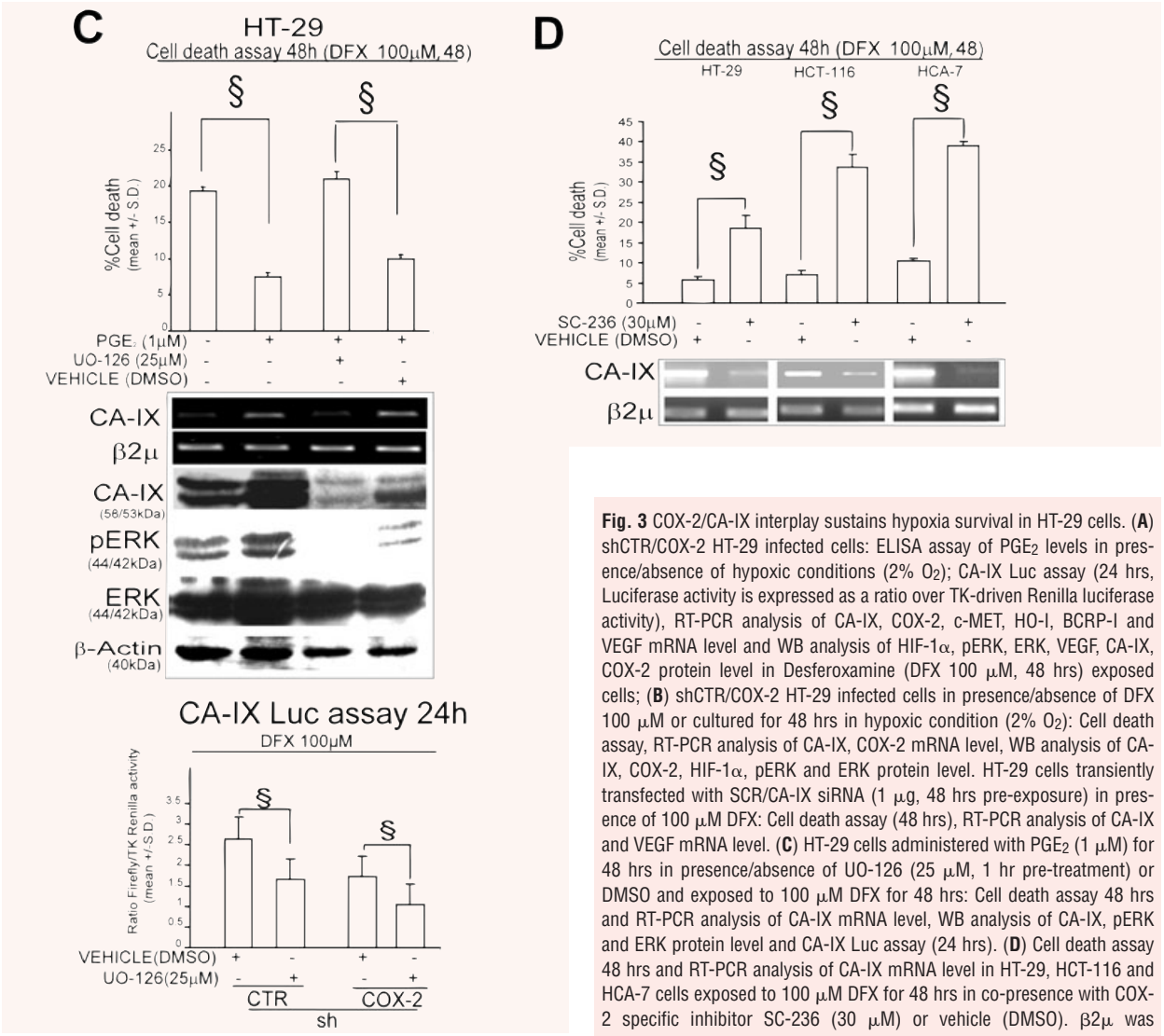


Fig. 3 COX-2/CA-IX interplay sustains hypoxia survival in HT-29 cells. (**A**) shCTR/COX-2 HT-29 infected cells: ELISA assay of PGE_2 levels in presence/absence of hypoxic conditions (2% O_2); CA-IX Luc assay (24 hrs, Luciferase activity is expressed as a ratio over TK-driven Renilla luciferase activity), RT-PCR analysis of CA-IX, COX-2, c-MET, HO-I, BCRP-I and VEGF mRNA level and WB analysis of HIF-1α, pERK, ERK, VEGF, CA-IX, COX-2 protein level in Desferoxamine (DFX 100 μM, 48 hrs) exposed cells; (**B**) shCTR/COX-2 HT-29 infected cells in presence/absence of DFX 100 μM or cultured for 48 hrs in hypoxic condition (2% O_2): Cell death assay, RT-PCR analysis of CA-IX, COX-2 mRNA level, WB analysis of CA-IX, COX-2, HIF-1α, pERK and ERK protein level. HT-29 cells transiently transfected with SCR/CA-IX siRNA (1 μg, 48 hrs pre-exposure) in presence of 100 μM DFX: Cell death assay (48 hrs), RT-PCR analysis of CA-IX and VEGF mRNA level. (**C**) HT-29 cells administered with PGE_2 (1 μM) for 48 hrs in presence/absence of UO-126 (25 μM, 1 hr pre-treatment) or DMSO and exposed to 100 μM DFX for 48 hrs: Cell death assay 48 hrs and RT-PCR analysis of CA-IX mRNA level, WB analysis of CA-IX, pERK and ERK protein level and CA-IX Luc assay (24 hrs). (**D**) Cell death assay 48 hrs and RT-PCR analysis of CA-IX mRNA level in HT-29, HCT-116 and HCA-7 cells exposed to 100 μM DFX for 48 hrs in co-presence with COX-2 specific inhibitor SC-236 (30 μM) or vehicle (DMSO). β2μ was assessed as quantitative control for RT-PCR analysis. β-Actin was assessed as quantitative control for WB analysis. Data are expressed as mean ± S.D. of three replicates ($n = 3$) (ANOVA test, $^{\S}P < 0.001$, *post hoc* test adjustment for multiple comparison was applied when required).

in HT-29, HCT-116 and HCA-7 cells exposed to DFX (100 μM, 48 hrs) (Fig. 3D). Notably, no cell death was observed in cells treated with SC-236, in absence of DFX (100 μM for 48 hrs) (data not shown). These data support the argument that COX-2/CA-IX interplay sustains hypoxia survival in CRC cells.

High-density culture condition up-regulates COX-2/CA-IX interplay

High-density culture conditions associate with CA-IX gene up-regulation, as well as promote pericellular hypoxic environment [21, 37, 38]. We found an increase of COX-2/pERK/CA-IX protein level in HT-29 cells cultured at high (2×10^5cells/cm^2) respect to low (0.4×10^5cells/cm^2) density (Fig. 4A). We also observed a cell density-dependent increase of the invasive potential in shCTR, but not in shCOX-2 HT-29 infected cells (Fig. 4B). As expected, the increase in the invasive capacity of HT-29 cells cultured at high density was blocked by CA-IX, but not SCR siRNA transfection (Fig. 4C). In the same culture condition, no cell death was observed in shCOX-2 infected or CA-IX siRNA transfected cells (data not shown). Overall, the data reported show that COX-2/CA-IX interplay is up-regulated and modulates invasive behaviour in mild hypoxic conditions.

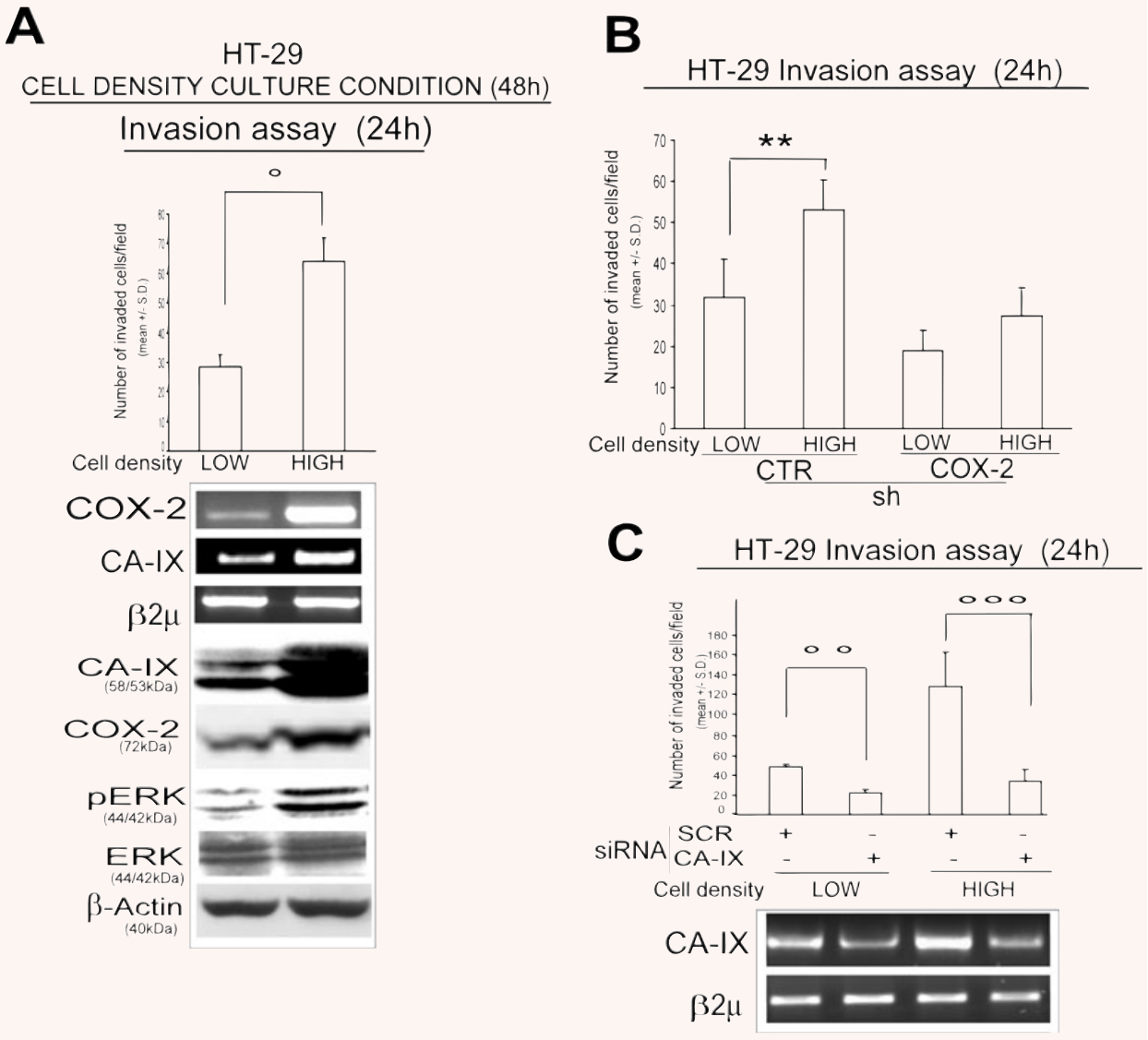


Fig. 4 The COX-2/CA-IX up-regulation promotes invasive potential of colorectal cancer cells in high-density culture condition. (**A**) HT-29 cells cultured at low (0.4×10^5 cells/cm^2) and high (2×10^5 cells/cm^2) density condition for 48 hrs: Boyden chamber invasion assay (24 hrs), RT-PCR analysis of COX-2 and CA-IX mRNA level and WB analysis of CA-IX, COX-2, pERK, total ERK protein level. (**B**) Boyden chamber invasion assay (24 hrs) of shCTR/COX-2 HT-29 infected cells cultured at low- and high-density condition. (**C**) HT-29 cells cultured at low- and high-density condition and transiently transfected with SCR and CA-IX siRNA (1 μg, pre-exposure for 48 hrs): Boyden chamber invasion assay (24 hrs) and RT-PCR analysis of CA-IX mRNA level. β2μ was assessed as quantitative control for RT-PCR analysis. β-Actin was assessed as quantitative control for WB analysis. Data are expressed as mean ± S.D. of three replicates ($n = 3$) (ANOVA test, $^{**}P = 0.006$, $^{\circ}P = 0.003$, $^{\circ\circ}P = 0.01$, $^{\circ\circ\circ}P < 0.001$).

High COX-2 and CA-IX protein expression correlates with tumour stage in CRC specimens

The results above suggest us that the COX-2/CA-IX interplay controls malignant features of CRC cells. We then assessed the expression of such genes in tissues from CRC patients, by quantitative evaluation of IHC and WB analyses. IHC analysis revealed a significant correlation between COX-2 and CA-IX protein expression for all stages (Spearman correlation coefficient: $\rho = 0.500$, $P = 0.0005$), being the correlation present also when stages were analysed separately (Spearman correlation coefficient: Stage II $\rho = 0.640$, $P = 0.0004$ (Fig. 5A); Stage III $\rho = 0.366$, $P = 0.04$; Stage IV $\rho = 0.847$, $P = 0.016$) except for Stage I (Spearman correlation coefficient: Stage I

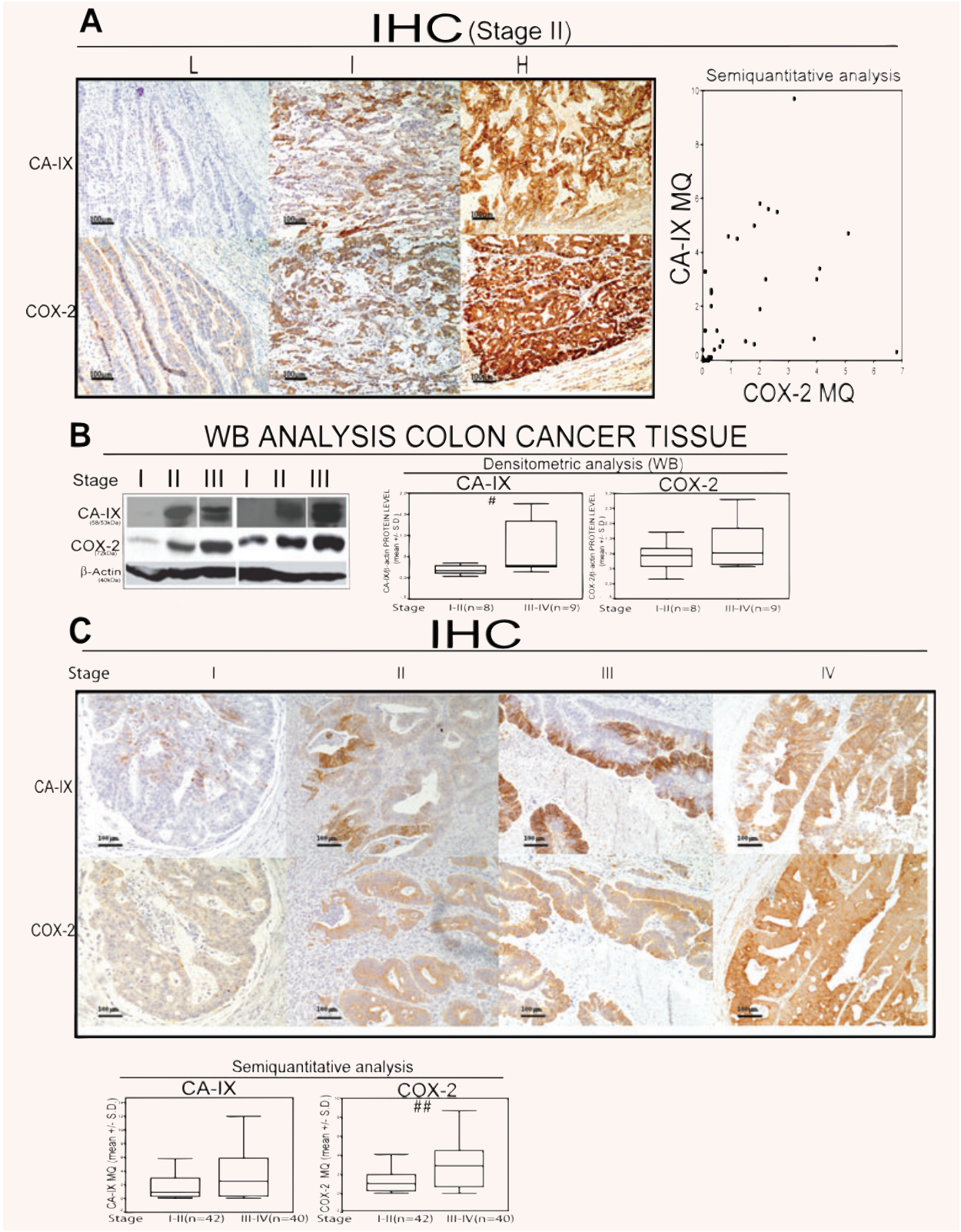


Fig. 5 COX-2 and CA-IX protein level correlate with CRC stage. (**A**) Representative IHC in stage II CRC tissue expressing low (L), intermediate (I) and high (H) CA-IX/COX-2 proteins, scatter plot of immunohistochemical (IHC) semi-quantitative evaluation (Multiplicative Quick score, MQ) of CA-IX and COX-2 protein expression in stage II, CRC ($n = 35$); (**B**) WB analysis of CA-IX and COX-2 protein level in 6 representative CRC tumour tissue samples and densitometric analysis of CA-IX and COX-2 protein level assessed by WB analysis in 17 CRC tumour tissue samples (Mann–Whitney test: stage I-II *versus* stage III-IV: CA-IX $^{\#}P = 0.042$; COX-2 $P = 0.48$). β-Actin was assessed as quantitative control for WB analysis; (**C**) Representative IHC analysis of CA-IX and COX-2 protein expression level and IHC semi-quantitative evaluation of CA-IX and COX-2 protein expression level in a cohort of 82 CRC tissues (Mann–Whitney test: CA-IX MQ, $P = 0.14$; COX-2 MQ, $P = 0.0011$; stage I-II *versus* stage III-IV: CA-IX MQ, $P = 0.075$; COX-2 MQ, $^{\#\#}P = 0.003$).

$\rho = 0.307$, $P = 0.460$) (Fig. 5A). WB and IHC analyses revealed an increase of COX-2 and CA-IX protein expression level in stage III-IV CRC compared to stage I-II CRC (Figs. 5B, C and S2). These data indicate that COX-2/CA-IX protein expression in CRC specimens is correlated and increased with tumour stage.

Discussion

In this investigation, we show that, in CRC cells, the pro-inflammatory enzyme COX-2 up-regulates the expression of the hypoxia response gene CA-IX, throughout the PGE_2-dependent activation of ERK-1/2. In CRC cells, COX-2/CA-IX axis elicits aggressive features (invasive behaviour and hypoxia survival). Such activities have been previously associated to the expression of these genes in separate experimental models [12, 23]. Here, we provide evidence for the existence of a COX-2/CA-IX axis that promotes malignant behaviour in CRC cells.

CA-IX gene expression has been previously shown to be induced by HIF-1α transcription factor and ERK activation [12, 21, 22]. Here, we show that the up-regulation of COX-2 expression upon hypoxia exposure contributes to the ERK-dependent up-regulation of CA-IX expression. The ERK-dependent regulation of CA-IX gene expression has been observed in cells cultured at high-density culture condition that creates a hypoxia-like environment, by decreasing pericellular pO_2 [37, 38]. Accordingly, we show that, in dense culture condition, COX-2 up-regulation promotes CA-IX gene expression and invasive behaviour. These data indicate that hypoxia and mild hypoxic environment, generated by cell overgrowth, trigger COX-2/CA-IX axis to enhance malignant features in CRC cells.

CA-IX gene expression contributes to the acidification of the extracellular environment by catalysing the hydration of carbon dioxide to bicarbonate and protons, a phenomenon that induces the activation of extracellular matrix enzymes, MMPs [36]. Noteworthy, PGE_2 production and ERK activation have been previously demonstrated to enhance invasiveness and MMPs activity [31, 32]. We here report that COX-2/CA-IX axis up-regulation increases the activity of MMP-2, an enzyme linked to CRC malignancy [39].

CA-IX protein expression is regarded as surrogate marker of hypoxia *in vivo* [17]. The expression of CA-IX correlates with poor prognosis in several cancers [18, 19]. In CRC tissues, CA-IX gene expression has been previously associated with cell proliferation status [20, 40]. In this investigation, we show that CA-IX expression in CRC tissues correlates with stage, thus suggesting that the enzyme expression increases with the extent of tumour aggressiveness. COX-2 expression has been previously correlated with poor prognosis in CRC [6, 41]. It may be therefore proposed to jointly evaluate COX-2 and CA-IX expression in cohorts of CRC patients in which long-term survival is available, in order to verify whether combination of these markers may identify subsets of patients with different risk of relapse.

COX-2 is a major player of inflammation [6]. Inflammatory molecules play a crucial role in CRC growth and malignancy and inflammatory cells promote an aggressive behaviour in CRC cells [42–44]. Based on the data presented, it may be proposed that the CA-IX-dependent aggressive behaviour is also under the control of inflammation. Such an inflammation-driven CA-IX gene up-regulation may explain the poor *in vivo* correlation occurring between CA-IX protein expression and the presence of hypoxia in CRC tissues [20]. Speculatively, CA-IX *in vivo* expression may be a marker of COX-2 activation as a consequence of the exposure of CRC cells to a variety of environmental conditions such as hypoxia, cell overgrowth and inflammation.

COX-2 inhibitors have been proposed as therapeutic tools for CRC patients [45]. A plenty of CA-IX inhibitors have been synthesized so far [46]. Our data suggest that the pharmacological down-regulation of COX-2/CA-IX axis may be a strategy to counteract the aggressiveness of CRC cells [47–49].

In conclusion, the data here presented indicate that the aggressive phenotype of CRC cells is controlled by a pathway, which can be up-regulated by two environmental conditions that have been linked to CRC growth and malignancy, *i.e.* inflammation (COX-2) and hypoxia (CA-IX). Studies regarding the role of this tight relationship in CRC prognosis and therapy response are warranted.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Fig. S1. COX-2/CA-IX up-regulation sustains hypoxia survival in colorectal cancer cells. (**A**) HCT-116 cells transiently transfected with CTR/COX-2 siRNA (1 mg, 48 hrs pre-exposure) and HCA-7 cells stably infected with shCTR/COX-2 pSUPER.retro vector exposed to 100 mM DFX for 48 hrs: Cell death assay 48 hrs and RT-PCR analysis of CA-IX and COX-2 mRNA level. (**B**) HCT-116 cells pre-treated with PGE_2 1 mM for 24 hrs and/or transiently transfected with CA-IX/SCR siRNA (1 mg, 24 hrs pre-exposure) and then exposed to DFX 100 mM for 48 hrs: Cell death assay 48 hrs and RT-PCR analysis of CA-IX mRNA level. Data are expressed as mean ± S.D. of three replicates ($n = 3$) (ANOVA test, $^{\S}p < 0.001$, *Post Hoc* test adjustment for multiple comparison was applied when required).

Fig. S2. COX-2/CA-IX expression in CRC tissues. Representative immunohistochemical staining of COX-2 and CA-IX protein level in stage I-III-IV. CRC cancer tissue samples (scale bar, 500 mm, 100 mm, 20 mm). IHC photos represent both the mass of the tumour (M) and the tumour host interface (THI).

Table S1. Western blot antibodies and conditions. Legends: TBS-TB buffer (20 mmol/L Tris, pH 7.6, 150 mmol/L NaCl, 0.1% Tween 20, 5% bovine serum albumin) TBS-TM buffer (20 mmol/L Tris, pH 7.6, 150 mmol/L NaCl, 0.1% Tween 20, 5% non-fat dry milk).

Table S2. Primers sequences and conditions. Legends: COX-2: cyclooxygenase-2; CA-IX: carbonic Anhydrase-IX; b_2m: B-2 microglobulin; C-MET: Hepatocyte Growth Factor receptor; VEGF: vascular endothelial growth factor; HO-1: Heme oxygenase-1; BCRP-1: Breast Cancer Resistance Protein-1.

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