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Heme oxygenase-1 has antitumoral effects in colorectal cancer: Involvement of p53

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ABSTRACT

The expression of heme oxygenase-1 (HO-1) has been shown to be up-regulated in colorectal cancer (CRC), but the role it plays in this cancer type has not yet been addressed. The aims of this study have been to analyze HO-1 expression in human invasive CRC, evaluate its correlation with clinical and histo-pathological parameters and to investigate the mechanisms through which the enzyme influences tumor progression. We confirmed that HO-1 was over-expressed in human invasive CRC and found that the expression of the enzyme was associated with a longer overall survival time. In addition, we observed in a chemically-induced CRC animal model that total and nuclear HO-1 expression increases with tumor progression. Our investigation of the mechanisms involved in HO-1 action in CRC demonstrates that the protein reduces cell viability through induction of cell cycle arrest and apoptosis and, importantly, that a functional p53 tumor suppressor protein is required for these effects. This reduction in cell viability is accompanied by modulation of the levels of p21, p27, and cyclin D1 and by modulation of Akt and PKC pathways. Altogether, our results demonstrate an antitumoral role of HO-1 and points to the importance of p53 status in this antitumor activity.

1. Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and the second in females (Ferlay et al., 2010). Histopathological staging from microscopic examination of tumor tissue is the standard for diagnosis and prognosis, as well as being the main guide for the choice of treatment (Compton and Greene, 2004). However, tumors with the same histo-pathological classification may display significant differences in progression and response to treatment (Liefers and Tollenaar, 2002). This reflects the need to identify new molecular markers that help in the diagnosis, prognosis and treatment of the disease.

Heme oxygenase (HO) is a microsomal enzyme catalyzing the first rate-limiting step in heme degradation, leading to the formation of equimolar quantities of carbon monoxide, biliverdin and free iron. HO-1, the inducible 32-kDa isoform, is an ubiquitous heat shock protein (HSP32) (Maines and Gibbs, 2005) that can be induced in response to cellular stress, oxidative stimuli and hypoxia, an important process frequently occurring during tumor growth. An increasing body of evidence indicates that HO-1 may play an important role in cancer. Indeed, HO-1 was reported to be up-regulated in rat, mouse and human tumors (Jozkowicz et al., 2007; Was et al., 2006), although the significance of this up-regulation is not clear. In this regard, we have recently demonstrated that HO-1 protein is overexpressed and correlates with clinical parameters in head and neck squamous cell carcinoma (Gandini et al., 2012), glioma (Gandini et al., 2014) and in non-small cell lung cancer (Degese et al., 2012) and that the nuclear localization of the protein associates with tumor progression (Gandini et al., 2012). Several groups have studied the expression of the enzyme in intestinal diseases such as colitis (Berberat et al., 2005; Takagi et al., 2008), inflammation (Barton et al., 2003) and inflammatory bowel disease (Paul et al., 2005). In both the normal intestinal physiology and intestinal diseases, HO-1 has been shown to be increased in response to oxidative stress (Degese et al., 2012) and to play an important role in mucosal protection by scavenging free radicals and reducing inflammation (Berberat et al., 2005). To our knowledge, there are only three reports in the literature showing the expression of HO-1 in human CRC samples (Becker et al., 2007; Kang et al., 2012; Yin et al., 2014).

All this background suggested the need to address the role of HO-1 in CRC and for this purpose we evaluated enzyme expression in human CRC tissues and analyzed its correlation with clinic-pathological features. Additionally, and since the function of HO-1 in CRC biology is

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far from being completely understood, we have also begun to study the mechanisms through which the aforementioned protein influences colorectal tumor progression.

2. Materials and methods

2.1. Patients and tissue specimens

83 human CRC samples were retrieved from the Hospital Regional Italiano (Bahía Blanca, Argentina) with institutional approval. These samples corresponded to primary tumors obtained by surgical resection of invasive CRC patients. The staging of CRC was classified using the seventh edition of the International Union Against Cancer Tumor-Node-Metastasis (TNM) staging system. Data were obtained on diagnosis, treatment response, course and follow-up, gender, primary tumor site, date of surgery, date of death or last contact, date of relapse, site of relapse, obstruction and/or intestinal perforation at diagnosis and presurgical carcinoembryonic antigen (CEA). From the primary tumor biopsy we obtained tumor size, invasion to the intestinal wall, regional lymph node metastasis, vascular or perineural invasion, grade of differentiation, K-ras status and expression of EGFR-1 receptor. Additionally, a cohort of 15 samples was retrieved from private pathology laboratories. H&E staining was performed on each sample and the slides further re-evaluated by a pathologist. A series of 5-μm sections were cut and transferred onto histological glass slides.

2.2. Immunohistochemistry (IHC)

The tissues obtained were fixed for 24 h in 10% formalin and embedded in paraffin using standard procedures. Then the slides were treated as previously described (Facchinetti et al., 2010; Gandini et al., 2012). Sections were then incubated overnight at 4 °C with primary rabbit anti-HO-1 antibody (SPA-896, Streesgen; 1:100) followed by incubation for 30 min with diluted biotinylated secondary antibody and then incubation with Vectastain ABC Reagent (Vector Laboratories Inc.). For negative controls, the primary antibody was omitted. Diaminobenzidine/H_2O_2 was used as substrate for the immunoperoxidase reaction and the tissues were lightly counterstained with hematoxylin (Harris), dehydrated through grade ethanol and xylene and mounted with Permount (Fischer Scientific) for analysis by bright-field microscopy.

2.3. Evaluation of staining intensity and statistical analysis

Human immunostained sections were scored semiquantitatively based upon the proportion of tumor cells stained and the staining intensity, by using the semi-quantitative immunoreactive score (IRS), as previously described (Gandini et al., 2012, 2014). To estimate the discriminative value of the IRS for HO-1 expression in CRC, receiver operating characteristic (ROC) curves were plotted and the corresponding areas under the curves (AUCs) were compared using various possible cut-off values, as already described (Gandini et al., 2014). In the animal model, the percentage of HO-1 expression was analyzed by counting cells in 10 random fields (400×). To study HO-1 nuclear staining in human samples and in the animal model, the total percentage of nuclei positive for HO-1 was assessed. Samples that had more than 10% of stained cells were considered positive. The software Graph Pad Prism 5 was used for the collection, processing and statistical analysis of all data. The statistical significance of HO-1 expression between groups was determined by the two-tailed χ^2 test or Mann–Whitney *U* test. *p* values of less than 0.05 indicated a significant result.

2.4. Animal model

Wistar male rats (N = 31) aged 8 weeks old were used. 28 of them were injected intramuscularly with 1,2-dimethylhydrazine (DMH) 20 mg/kg once a week for 8 weeks. At progressive stages of the development of tumors, animals were anesthetized and sacrificed to collect the necessary samples (polyps, adenocarcinomas and signet-ring cell carcinomas). Normal tissue was collected from animals that belonged to the same colony but were not treated with DMH (n = 3), in order to perform comparative studies with normal mucosa. Samples were taken for fixing in 10% formalin for immunohistochemistry. The statistical significance of HO-1 expression between groups was determined by the two-tailed χ^2 test or Mann–Whitney *U* test. *p* values of less than 0.05 indicated a significant result.

2.5. Cell lines

Human CRC cell lines HCT116, HCT116 p53 −/−, HT29 and LoVo were maintained at 37 °C in a humidified incubator with 5% CO_2/95% air atmosphere in DMEM and F-12K Medium (Sigma) supplemented with 10% (v/v) FBS (Gibco), L-glutamine (5 mM, Gibco), penicillin (Gibco, 100 U/ml) and streptomycin (Gibco, 100 μg/ml).

2.6. Flow cytometry

For cell cycle analysis staining with propidium iodide (PI, Sigma) was used. 1×10^6 cells HCT116 and HCT116 p53 −/− were seeded. The cells were synchronized by deprivation of fetal bovine serum for 24 h. Then they were treated with hemin and vehicle (100 μM) for 24 h. The cells were trypsinized, fixed with ice-cold 70% ethanol, stained with PI, and analyzed for DNA content by FACScan flow cytometry (Becton Dickinson, Germany). Data were analyzed by Cell Quest software (Becton Dickinson). The percentage of apoptotic cells was measured by flow cytometry following Annexin V (FL1-H) and PI (FL2-H) labeling. All these experiments were carried out in triplicate and were repeated twice.

2.7. Transient transfections

We used two expression plasmids for HO-1 over-expression. One encoding the native form of the protein fused to enhanced green fluorescent protein (pEGFP-HO-1) was kindly donated by Dr. Phyllis A. Dennery from the Children's Hospital of Philadelphia, University of Philadelphia, USA. The other, pcDNA3-HO-1 was gently donated by Dr. Elba Vazquez (Universidad de Buenos Aires). Also, a pcDNA3–p53, kindly donated by Adriana De Siervi (Universidad de Buenos Aires) was employed to introduce p53 in the HCT116 p53 −/− cell line. The transfection procedure was performed by using Lipoafectamine (Invitrogen, CA, USA) according to the manufacturer's instructions.

2.8. Cellular viability assays

HCT116, HCT116 p53 −/− and HT29 CRC cell lines were seeded in 96 well plates. 48 h later, they were treated with an inductor or an inhibitor of the activity of HO-1 (hemin and tin dichloride (IV) protoporphyrin (IX) (SnPP), respectively) at different doses and different time points after which the cells were incubated with WST-1 cell proliferation reagent (Roche) and further counted manually using a hemocytometer (Becton Dickinson, Germany).

Alternatively, both HCT116 and HCT116 p53 −/− cell lines were seeded in 96 well plates and 72 h later the HCT116 p53 −/− cells were transfected as previously described. 24 h post-transfection the cells were treated with hemin (100 μM) and vehicle and 96 h later the cells were incubated with WST-1 and manually counted.

2.9. Cell migration

Cell migration was measured by a “wound healing” assay as previously described (Petit et al., 2000). Cells were seeded and further treated for 24 h with vehicle or hemin (100 μM).

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2.10. Cell lysis and western blot analysis

The entire procedure was performed as already described (Facchinetti et al., 2010). HCT116, HCT116 p53 −/− and HT29 cells were grown for 48 h. Following cell line treatment with hemin (20, 40 and 100 μM), SnPP (2.5 and 10 mM) and vehicle (DMSO), or cell line transfection as detailed previously, the cells were scraped and proteins were quantified using the Bradford method (Bradford, 1976). The lysates were electrophoresed and the blots were incubated with primary rabbit polyclonal anti-HO-1 antibody (SPA-896, Stressgen Bioreagents, Canada), mouse polyclonal anti-p27/Kip1 (BD Transduction Laboratories™), mouse polyclonal anti-p21 (BD Pharmingen™), rabbit monoclonal anti-cyclin D1 (SP4, Thermo Scientific, Inc.), rabbit polyclonals anti-p53 (sc-6243, Santa Cruz Biotechnology), anti-bax (sc-493, Santa Cruz Biotechnology, Inc.), anti-PKC β I (sc-209, Santa Cruz Biotechnology, Inc.), anti-PKC β II (sc-210, Santa Cruz Biotechnology) and goat polyclonal anti-actin (sc-1615, Santa Cruz Biotechnology). The blots were finally washed with PBS-T buffer, incubated with secondary horseradish peroxidase conjugated antibody (Santa Cruz Biotechnology) and the reaction was detected by chemiluminescence amplified (ECL, GE Healthcare UK Limited).

2.11. Immunofluorescence

HCT116 and HCT116 p53 −/− cells were plated on sterile glass coverslips. The latter were transfected with pcDNA3–p53 and pcDNA3 plasmids. 48 h later they were treated with hemin and vehicle (100 μM) and then were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.1% Triton X-100 (Sigma), followed by incubation with a blocking solution (1% BSA in PBS). Anti-HO-1 (SPA-896 Stressgen, dilution: 1:100) and anti-p53 (sc-6243, Santa Cruz Biotechnology, dilution: 1:100) were used. After incubation with primary antibody, cells were incubated with Alexa 566 fluoro-conjugated antibodies (Molecular Probes, Invitrogen). Nuclei were stained with DAPI and then mounted on slides with Prolong Gold anti-fade reagent (Invitrogen). Images were captured with a Nikon Eclipse E600 fluorescence microscope equipped with a Nikon camera. Counting of 200 cells in 10 random fields (400×) was done in order to study the proportion of cells containing HO-1 and p53 expression.

3. Results

3.1. Validation of the antibody for immunohistochemical analyses and HO-1 expression in human CRC biopsies

Immunospecificity of the antibody for HO-1 in human CRC was first tested by western blot of protein lysates of human CRC cell lines HT29 and HCT116 (Fig. 1A). The antibody exclusively recognized one band representing a protein with a mobility corresponding to the molecular weight of HO-1 (32 kDa). This antibody was then used for further studies of HO-1 expression in human CRC samples. We subsequently performed immunohistochemical staining in 98 surgically-resected biopsies of

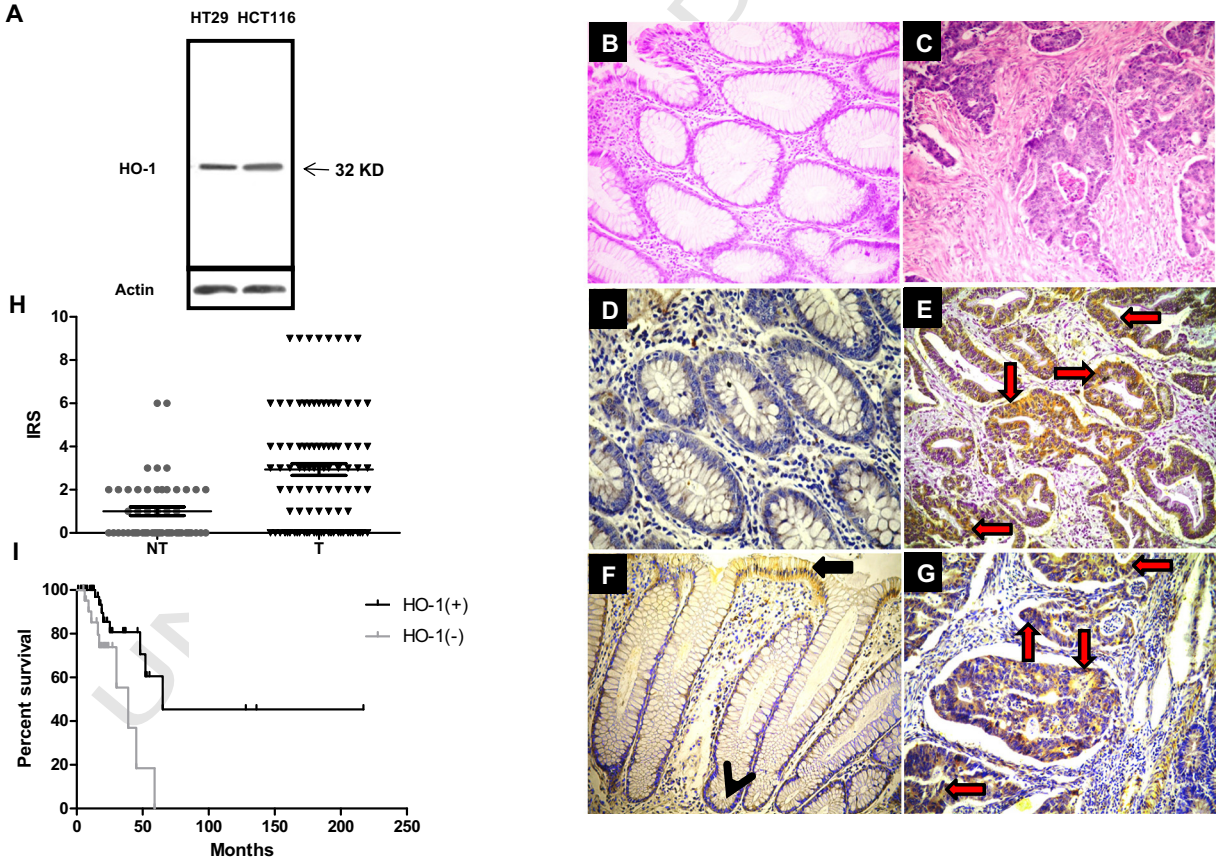

Fig. 1. Specific detection of HO-1 in human colorectal biopsies and association of HO-1 expression with longer overall survival times in patients with invasive CRC. Western blot analysis of HT29 and HCT116 cell lines (A) probed with anti HO-1(SPA-896). Hematoxylin and eosin staining of histologically normal tissue adjacent to the tumor (B) and of tumor tissue (C) (40×). HO-1 expression in non-malignant adjacent tissues (D and F) (100 and 40×) and in their respective tumor tissues (E and G) (100×). Black arrow: HO-1 immunoreactivity observed in apical cells of the crypt within the non-malignant epithelia. Arrowhead: basal cells lacking HO-1 staining. Red arrows: High HO-1 expression in tumor tissues. H. Dot plot showing the levels (IRS) of HO-1 in tumor tissues (T) and in adjacent non-malignant tissues (NT). Immunohistochemical analysis revealed differences in the levels of HO-1 expression between adjacent non-tumor tissues (NT) and tumor tissues (T) ($p = 0.0001$, Mann Whitney test). I. Kaplan Meier survival plots. Patients with positive HO-1 expression in their tumors present a longer OS time than patients displaying negative HO-1 expression ($N = 74$; $p = 0.002$, log-rank test). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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invasive CRC. Staining with hematoxylin & eosin to each slide was first done to study histopathological characters in order to confirm the diagnosis (representative samples: Fig. 1B and C). Thirty two of the samples contained histologically-normal tissues (non-malignant epithelia) adjacent to the tumor tissues. We then proceeded with the immunohistochemistry for HO-1 as described in the Materials and methods section. HO-1 immunoreactivity was observed in apical cells of the crypt within the non-malignant epithelia (Fig. 1F, black arrow), and basal cells showed no HO-1 staining (Fig. 1F, red arrow). Tumor specimens showed higher rates of expression (69.3%, 68/98; Fig. 1E and G) than their respective surrounding non-malignant tissues (12.5%, 4/32; $p = 0.04$; Fig. 1D and F and Supplementary Table 1). The analysis of HO-1 expression levels also showed differences between tumor (T) and adjacent non-malignant tissues (NT) (median IRS: 3 versus median IRS: 1, respectively; $p = 0.0001$; Fig. 1H).

3.2. HO-1 is associated with increased overall survival time of invasive CRC patients

We subsequently studied the correlation between HO-1 protein expression and several clinic-pathological parameters important for CRC prognosis such as gender, tumor location, differentiation grade, mitotic index, nuclear index, lymph node involvement, the presence of metastasis, K-ras status, pre-surgical CEA levels and EGFR expression (Table 1). This analysis revealed a significant correlation between HO-1 positive expression and wild-type K-ras status ($p = 0.04$) and normal CEA levels ($p = 0.04$, χ^2 test).

Our next step was to examine whether HO-1 could be considered a prognostic factor in invasive CRC, and for this purpose we analyzed if HO-1 protein expression was associated with patient overall survival time. For this analysis and in order to avoid the problems of multiple cut-point selection, ROC curve analysis was performed to determine a reasonable cut-off point of HO-1 in the CRC samples. The best HO-1 cut-off point for overall survival (OS) was a score ≥ 1 (sensitivity: 61.11 and specificity: 69.09; AUC = 0.703; 95% confidence interval: 0.585–0.804; $p = 0.0018$). In brief, patients with a cut-off score ≥ 1 were regarded as HO-1 positive and those with a score <1 were regarded as HO-1 negative.

Interestingly, we found an association between tumor HO-1 positivity and a longer patients' survival time (Fig. 1I; $p = 0.002$, log rank test). The patients whose tumors presented HO-1 positive expression had a median survival time of 65 months whereas the patients presenting negative expression of the protein had a median survival time of 39 months.

3.3. An increase in HO-1 expression and nuclear localization is associated with malignant progression in a CRC animal model

In order to gain insight into the significance of HO-1 in CRC, we assessed the expression of HO-1 during the progression of a chemically-induced CRC model. For this purpose, we isolated normal epithelial tissues (Fig. 2A and E), polyps (Fig. 2B and F), adenocarcinoma tissues (Fig. 2C and G) and signet-ring cell carcinoma tissues (Fig. 2D and H) that were obtained at different times of disease progression. Signet-ring cell carcinomas have been characterized as very malignant entities (Nissan et al., 1999; Bradford, 1976). We found positive staining in 2/10 (20%) of the normal tissue, 3/3 (100%) of the polyps, 12/12 (100%) of adenocarcinoma specimens and 3/3 (100%) of signet-ring cell carcinoma samples. Protein expression showed similar immune-staining pattern and sub-cellular localization to those observed in the human samples. Polyps ($p = 0.008$), adenocarcinoma ($p < 0.001$) and signet cell carcinoma ($p = 0.009$) showed higher rates of HO-1 protein expression than normal epithelia (Fig. 2I). HO-1 has been originally described as a microsomal enzyme. However an increasing number of reports (Sacca et al., 2007; Gueron et al., 2009; Birrane et al., 2013; Yin et al., 2014) including ours (Gandini et al., 2012, 2014) showed nuclear localization of the enzyme in some tissues and cell types and demonstrated an association of nuclear localization with the progression of the disease (Gandini et al., 2012). Therefore we studied HO-1 sub-cellular localization in tissues from this animal model of CRC. Nuclear staining in 3/3 normal tissues (mean percentage of stained nuclei of all the positive samples: 7.3 ± 2%), 3/3 of the polyps (15 ± 3%; $p = 0.035$), 8/12 of adenocarcinoma samples (15 ± 5%; $p = 0.019$) and 3/3 of signet cell carcinoma (25.6 ± 3%; $p = 0.002$) was observed, thus showing that HO-1 nuclear localization rates increase with disease progression ($p < 0.0001$, ANOVA; Fig. 2J).

Table 1
Summary of patient characteristics and HO-1 expression and correlation with clinic-pathological features.

	Cases	HO-1 positive n (%)	HO-1 negative n (%)	p
Gender				
Female	36	20(55.5)	16(44.5)	0.400
Male	39	25(64.1)	14(35.9)	
Not available	8			
Tumor location				
Colon	68	45(66.2)	23(33.8)	0.900
Rectus	15	10(66.7)	5(33.3)	
Not available	–			
Differentiation grade				
I	20	17(85)	3(15)	0.058
II	53	30(56.6)	23(43.4)	
III	5	4(80)	1(20)	
Not available	5			
Mitotic index				
I	20	16(80)	4(20)	0.100
II	32	18(56.25)	14(43.75)	
III	2	1(50)	1(50)	
Not available	29			
Nuclear index				
I	8	5(62.5)	3(37.5)	0.200
II	41	23(56.10)	18(43.9)	
III	4	4(100)	0(0)	
Not available	30			
Lymph node involvement				
No	17	12(70.6)	5(29.4)	0.400
Yes	46	28(60.87)	18(39.13)	
Not available	12			
K-ras status				
Wild type	55	36(65.45)	19(34.55)	0.040
Mutated	16	6(37.5)	10(62.5)	
Not available	12			
CEA levels				
Normal	20	15(70.6)	5(29.4)	0.040
High	26	12(46.2)	14(53.8)	
Not available	40			
EGFR1 positivity				
1+	17	8(47)	9(53)	0.100
2+	13	10(77)	3(23)	
3+	5	4(80)	1(20)	
Not available	48			

χ^2 test was used.

3.4. Nuclear localization of HO-1 is also observed in a CRC cell line and in human tissues

Since we had observed that nuclear localization was present and increased with tumor progression in the animal model of CRC, we also analyzed the presence of nuclear HO-1 by immunofluorescence in the human CRC cell line HCT116 and by immunohistochemistry in human CRC tissues. HO-1 expression was nuclear in HCT116 cells and increased with pharmacological activation (Fig. 3A). Additionally, all the adjacent non-malignant tissues that were positive for HO-1 showed cytoplasmic localization of the protein (100%) (Fig. 3B) whereas of the tumor samples

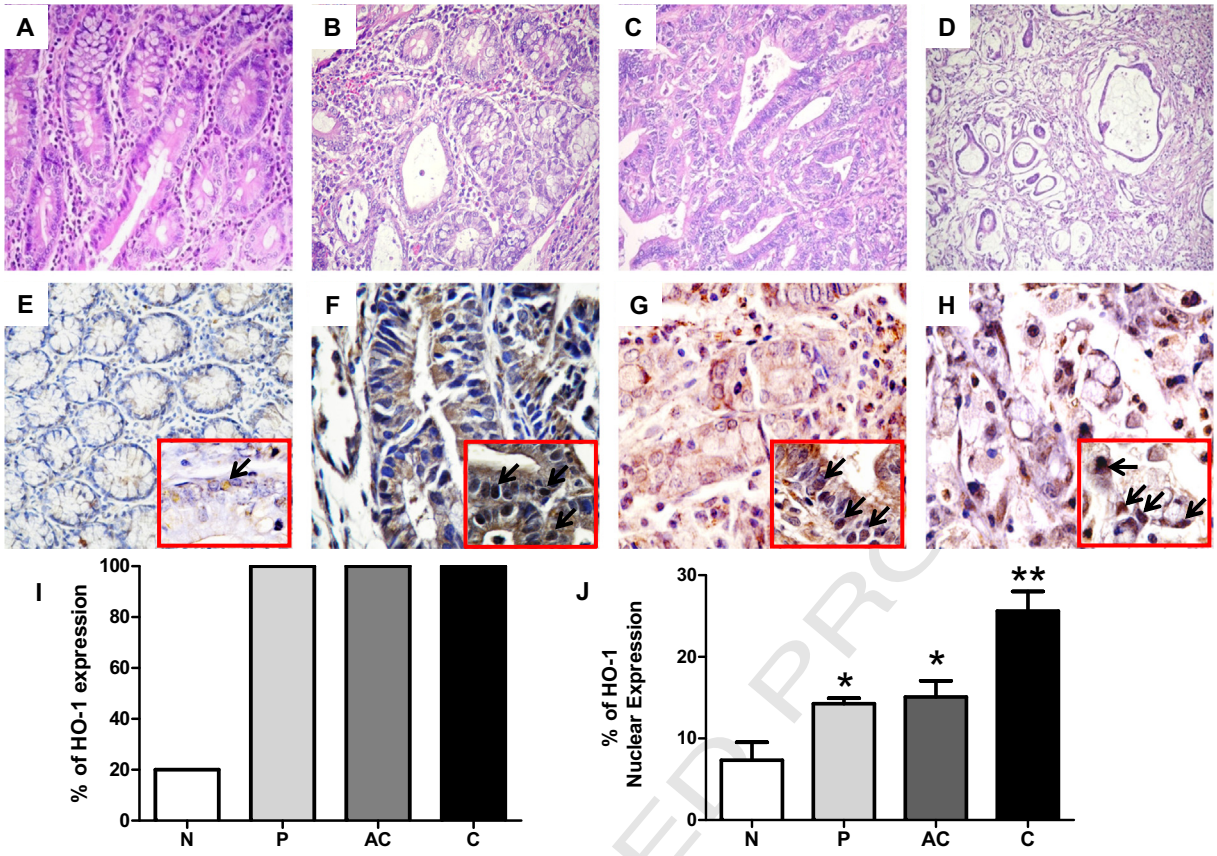

Fig. 2. HO-1 expression and nuclear localization increases with malignant progression in a chemically-induced animal model of CRC. Hematoxylin and eosin staining (A–D) (100×) and immunohistochemistry for HO-1 (E–H) (400 and 1000×) in non-malignant tissues (A and E), polyps (B and F), adenocarcinoma tissues (C and G) and signet-ring cell carcinoma tissues (D and H) obtained from the animal model. Black arrows show cytoplasmic (E) and nuclear (F–H) staining of HO-1. I. Analysis of HO-1 expression in non-malignant epithelia (N), polyps (P), adenocarcinoma (AC) and a signet-ring cell carcinoma (C). J. Graph showing the frequencies observed for the nuclear expression of HO-1 in non-malignant epithelia (N), polyps (P), adenocarcinoma (AC) and signet-ring cell carcinoma (C); $^{*}p = 0.035$, $^{*}p = 0.019$ and $^{**}p = 0.002$ compared to adjacent non-malignant tissues (N).

that were positive for HO-1, 61.8% (42/68) showed only cytoplasmic localization and 38.2% (26/68) showed both cytoplasmic and nuclear localization of the protein (Fig. 3C and D) ($p = 0.0001$, χ^2 test).

3.5. HO-1 decrease viability of cell lines that do not have alterations in the p53 gene

The HO-1 protein has been mainly described as having a protumoral activity in most types of cancers. However, as already mentioned, an antitumoral role has been suggested in prostate (Gueron et al., 2009) and colorectal cancers (Becker et al., 2007; Kang et al., 2012). The results obtained in human CRC biopsies corroborate that HO-1 expression is increased in tumors and that it is associated with a better patient outcome, thus suggesting an antitumoral activity for HO-1 in CRC. Therefore, in order to further investigate the role of HO-1 in CRC progression, we first evaluated the involvement of HO-1 on the viability of the colorectal cancer cell line HCT116. For this purpose, we first performed time-response analyses for cell viability after activation of HO-1 with hemin (100 μM) and observed that the number of cells decreased at 96 h of treatment ($p = 0.01$) (Fig. 4A). Taking this time point for future studies of cellular viability, we proceeded to perform dose–response studies with hemin and with the HO-1 inhibitor SnPP. A decrease in the viability of the cells was observed when doses of 40 μM and 100 μM of hemin were used (Fig. 4B). Contrariwise, an increase in cell number was observed when cells were treated with SnPP (10 μM) (Fig. 4C). Since the tumor suppressor p53 is important for the regulation of cellular survival and is frequently des-regulated in CRC, we performed similar experiments using the cell line HCT116 p53 −/− which lacks p53 protein. Interestingly, the effect of hemin and SnPP treatment on cellular survival was blunted (Fig. 4D and E). This difference observed between the two cell lines regarding their response to HO-1 up-regulation may be due to the presence of p53 acting through regulation of the expression of p21, an inhibitor of cyclin-dependent kinases (CDK's) and regulator of cell cycle progression. We therefore analyzed whether activation of HO-1 induced the expression of this and other proteins related to cell cycle arrest and cellular viability. As seen in Fig. 4F, modulation with hemin produced up-regulation of HO-1 in both cell lines (with and without p53), thus suggesting that the expression of HO-1 is independent of p53. Also hemin-up-regulation of HO-1 was accompanied with an increase in p21, p27 (Fig. 4G and H) and a decrease in cyclin D1 (not shown) in HCT116 cells. Instead, in the cell line lacking p53, overexpression of HO-1 was not accompanied by up-regulation of p21 or p27 (Fig. 4E and F). Time-response analyses performed with hemin revealed that p53 induction occurs simultaneously with HO-1 induction (Fig. 4I).

Since both HCT116 cell lines differ mainly in the presence or absence of p53, we inferred that this tumor suppressor might be involved in the effect of HO-1 on cell viability. Because of this, we additionally investigated the activation of HO-1 in two different CRC cell lines, the LoVo cell line that is wild type for p53 gene and the HT29 cell line that has a mutation at codon 273 of p53 gene. This mutation alters p53 sequences that are directly responsible for sequence-specific DNA binding, thus allowing the cell cycle to proceed unchecked (Oliver et al., 2010). We performed viability analyses after activation of HO-1 with hemin (100 μM). The analysis performed in LoVo cells showed that activation of HO-1 resulted in decreased cell number ($p = 0.037$) at 96 h post-

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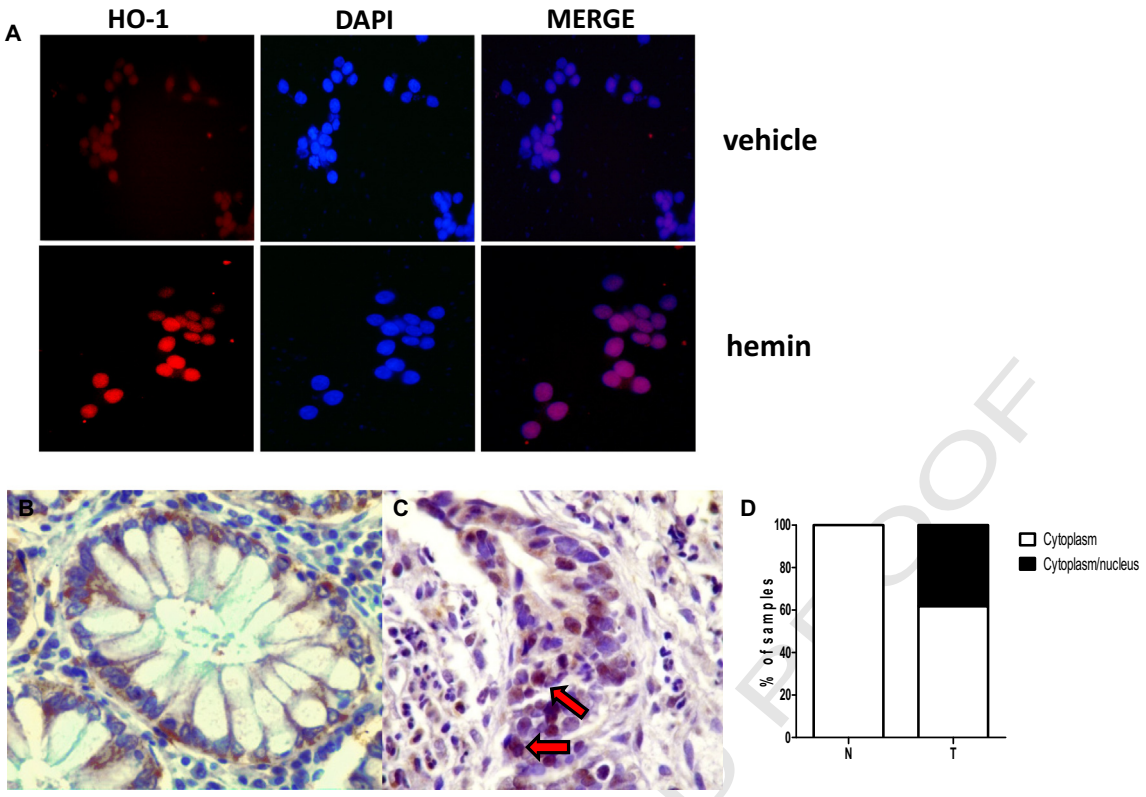

Fig. 3. Nuclear localization of HO-1 in HCT116 cell line and in human biopsies. A. HCT116 cells were treated with hemin (100 μM) or vehicle for 24 h and immunofluorescence for HO-1 was carried out. DAPI counter-stain was performed. B. The absence of nuclear localization of HO-1 in adjacent non-malignant human tissue. C. Nuclear localization of HO-1 in human tumor assayed by immunohistochemical staining (1000×). Red arrows show nuclear staining. D. Graph depicting nuclear and cytoplasmic rates of HO-1 in human tumor. 61.8% of samples showed cytoplasmic expression, while 38.2% showed both cytoplasmic and nuclear staining ($p = 0.0001$, χ^2 test). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

treatment (Fig. 5A). On the contrary, a time- and dose–response survival assay carried out in the HT29 cell line showed that the number of cells remained unchanged or slightly increased when treated with various doses of hemin at different time points (Fig. 5B). These results suggest that a functional p53 is necessary for HO-1-inhibition of cellular viability. This hypothesis was confirmed by re-expressing p53 tumor suppressor in HCT116 p53 −/− cell line by transient transfection of a pcDNA3 p53 plasmid and further performing viability analyses. We detected that the cell lines with altered p53 neither responded to HO-1 modulation nor showed a slight increase in cell survival, as previously observed (Fig. 5C; $p = 0.361$). However, when p53 was re-introduced in cells, their viability decreased after hemin treatment (Fig. 5C; $p = 0.0047$) presenting a similar behavior to p53-containing HCT116 cells ($p = 0.0006$). The efficiency of the transfection was analyzed by western-blot (Fig. 5D) and also by immunofluorescence (not shown), with 10% of HCT116 p53 −/− cells expressing p53 protein after transfection.

Since it has been demonstrated that HO-1 modulators display direct effects on some cellular processes that are not mediated by HO-1 activity (La et al., 2009) we proceeded to genetically over-express the enzyme in order to confirm the results obtained with pharmacological modulation of HO-1. We repeated the viability assay after genetic over-expression of HO-1 in the HCT116 and LoVo cell lines (Fig. 5E and F), obtaining similar results to those obtained by pharmacological overexpression of the protein ($p = 0.0017$ and $p = 0.0023$, respectively). The efficiency of the transfection was analyzed by immunofluorescence (50% of cells, data not shown).

The expression of cell survival-related proteins was analyzed by western-blot in HO-1-overexpressing HCT116 cells. Similar results were obtained than with the pharmacological induction of HO-1 (Fig. 5G).

3.6. The over-expression of HO-1 causes cell cycle arrest and apoptosis

We subsequently analyzed the possible mechanisms underlying HO-1 effects on cell viability. For this purpose, we studied if pharmacologic modulation of HO-1 induces cell cycle arrest by PI staining followed by flow cytometry. As observed in Fig. 6A, hemin treatment induces a G_0/G_1 arrest in HCT116 cell line ($p = 0.004$). No G_0/G_1 arrest was observed in HCT116 p53 −/− cells although a G_2/M arrest was induced (Fig. 6B; $p < 0.05$).

Subsequently, we considered if HO-1 activation could also regulate apoptosis in HCT116 cells and for this purpose labeling with Annexin V-fluorescein isothiocyanate (FITC) was performed. As shown in Fig. 6C, activation of HO-1 induces an increase in Annexin V staining in these cells ($p = 0.01$), thus suggesting that apoptosis is involved in HO-1 modulation of cellular viability.

In order to further analyze the mechanisms of HO-1-induced apoptosis we checked the expression of Bax, a pro-apoptotic protein that is known to be up-regulated by p53 (Chipuk et al., 2004). We observed an increase in the levels of this protein in the HCT116 cells when HO-1 was pharmacologically induced (Fig. 6D).

One common alteration in colon cancer is the hyper activation of the Akt and PKC (mainly β isoform) pathways. Therefore, we evaluated the activation state of Akt and expression of PKC following pharmacological modulation of HO-1. There was an increase in PKC βI levels in the parental cell line HCT116 when HO-1 was activated. In turn, there was a decrease of PKC βII following treatment with hemin, and an increase when SnPP was used (Supplementary Fig. 1). Importantly, a delay in the phosphorylation of Akt following HO-1 activation was observed in these cells (Fig. 6E). These results suggest that Akt and PKCβI pathways are involved in the effects observed on cellular survival.

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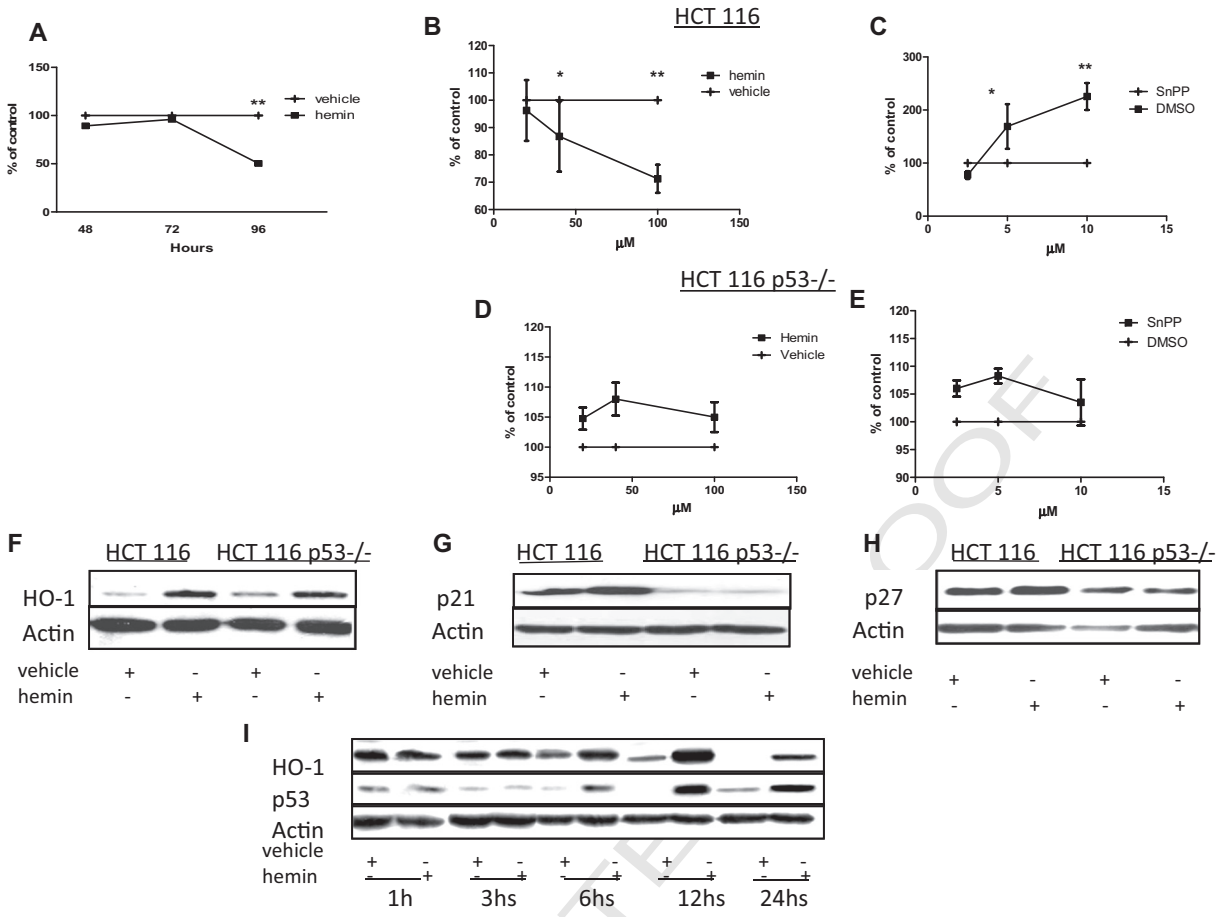

Fig. 4. Effect of pharmacological modulation of HO-1 on cell viability and on cell cycle-related protein expression in HCT116 and HCT116 p53 −/− cell lines. A. Time-response survival assay following hemin (100 μM) or vehicle treatment. The number of HCT116 cells decreased at 96 h. B–E. Dose–response viability assays following hemin (B and D) (20 μM, 40 μM or 100 μM) – or SnPP (C and E) (2 μM, 4 μM or 10 μM) – treatment in HCT116 (B and C) and HCT116 p53 −/− (D and E) cell lines. HCT116 and HCT116 p53 −/− cells were treated for 24 h with vehicle or hemin (100 μM) and protein lysates were electrophoresed and blotted against HO-1 (F), p21 (G) and p27 (H). I. HCT116 cells were treated for the times indicated, with vehicle or hemin (100 μM). The expression of HO-1 and p53 was analyzed by western-blotting. Actin was used as loading control.

3.7. HO-1 modulates cellular migration of HCT116 cell line

To evaluate the migratory capacity of HCT116 cells following HO-1 modulation, we used an in vitro scratch wound assay. Confluent monolayers of hemin-, vehicle- and SnPP-treated HCT116 cells were wounded. Wound closure was monitored every hour for 24 h as previously described. HCT116 cells treated with vehicle migrated and almost covered the wound by 24 h (uncovered wound area 60.28%), whereas in hemin-treated cells, a significant area of the wound (78.95%) remained uncovered over the same period. The inhibition of HO-1 with SnPP produced opposite results, that is the wound closed faster with SnPP than with the vehicle-treated cells (Supplementary Fig. 2). These results suggest that HO-1 is involved in modulating the migratory capacity of HCT116 cell line.

4. Discussion

In this study we have demonstrated that HO-1 is over-expressed in tumor epithelium of invasive CRC biopsies compared to their adjacent non-malignant epithelium. These results are in agreement with those obtained by our group in squamous cell carcinoma (Gandini et al., 2012), glioma (Gandini et al., 2014) and non-small cell lung cancer (Degese et al., 2012) where an up-regulation of HO-1 with tumor progression was found. Since induction of HO-1 is a fundamental cellular defense process against oxidative stress and other environmental insults, its increase in tumor cells may provide the first line of cellular defense of cancer cells against these insults. This might explain the increase in HO-1 expression observed in many different tumors. Indeed, HO-1 over-expression has also been demonstrated in lymphosarcoma (Schacter and Kurz, 1982), prostate carcinoma (Sacca et al., 2007) brain tumors (Deininger et al., 2000; Hara et al., 1996) renal carcinoma (Goodman et al., 1997), hepatoma (Doi et al., 1999), melanoma (Torisu-Itakura et al., 2000), Kaposi sarcoma (McAllister et al., 2004) pancreatic cancer (Berberat et al., 2005) and in chronic myeloid leukemia (Mayerhofer et al., 2004).

Regarding human CRC, a previous report showed apical staining in crypts of normal colonic epithelia, similar to our observations, and higher expression rates in CRC than in colon adenoma samples (Becker et al., 2007). Contrary to our results, they reported lower staining in high grade tumors. Two recent reports also showed higher expression levels in tumors (Kang et al., 2012; Yin et al., 2014). To our knowledge, no works have followed up with the study of HO-1 expression in human CRC. Instead, there are several studies addressing HO-1 function in both the normal intestinal physiology and inflammatory intestinal diseases. Interestingly, HO-1 expression is usually increased in gastrointestinal inflammation and injury, processes associated with cancer progression; this up-regulation was shown in gastric ulcers (Guo et al., 2002), colitis (Wang et al., 2001), radiation enteritis (Giriş et al., 2006), inflammatory bowel disease (Paul et al., 2005) both in patients and animal models. This up-regulation of HO-1 has been shown to play a role in protecting from inflammation and oxidative injury in the gastrointestinal tract (Zhu et al., 2011), a role that may inhibit CRC tumor progression and/or may improve patient outcome. According to

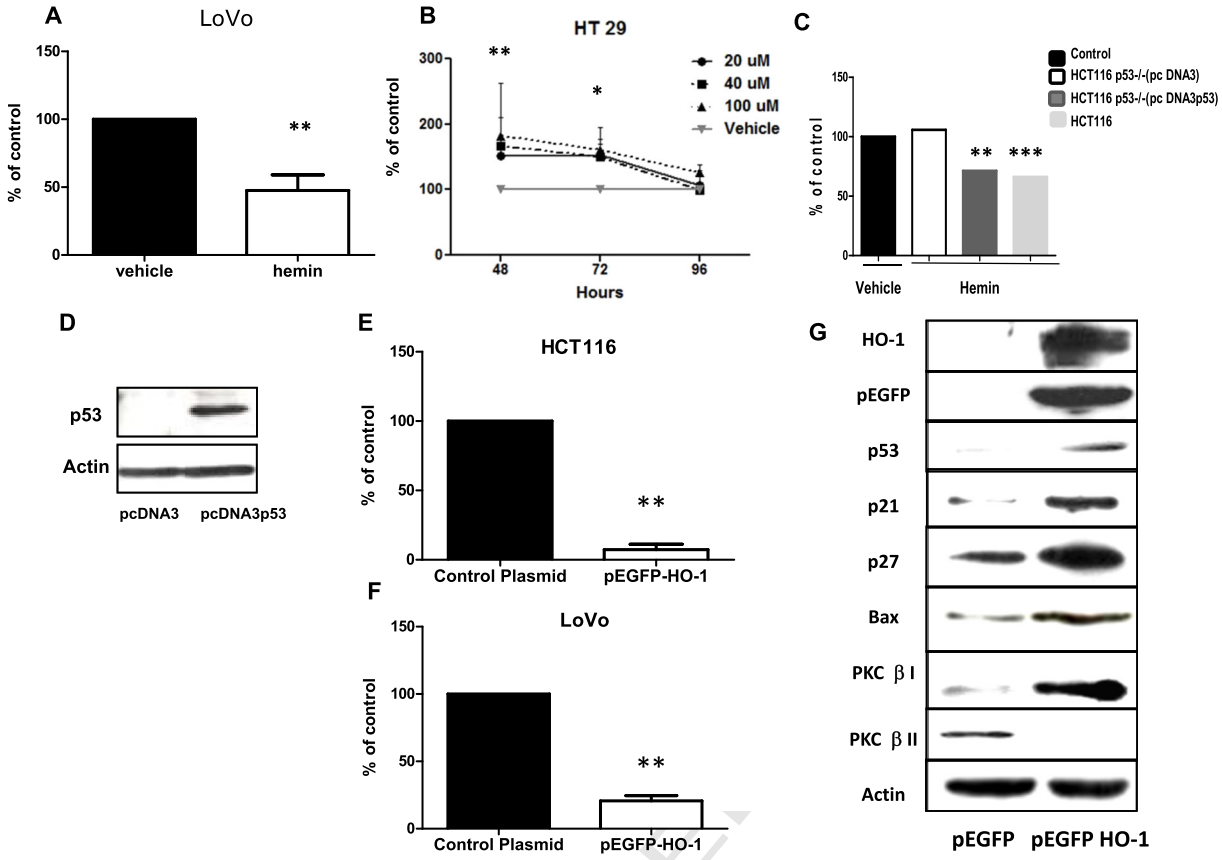

Fig. 5. Involvement of a functional p53 on HO-1 modulation of cell viability. A. Viability assay in the p53-wild type-containing LoVo cell line. Up-regulation of HO-1 with hemin (100 μM) decreased cell number ($p = 0.037$; *T* test). B. Viability assay in the p53-mutated-containing HT29 cell line. Dose (20, 40 and 100 μM) and time (48, 72 and 96 h)-response analysis. Hemin treatment leads to increased cell count at 48 and 72 hour treatment ($^{**}p = 0.01$, $^{*}p = 0.05$). C. Viability assays in HCT116 p53 −/− cell line transfected with pcDNA3 or pcDNA3 p53 and in HCT116 following hemin treatment ($^{**}p = 0.0047$, $^{***}p = 0.0006$). D. Western blot analysis showing re-expression of p53 in the HCT116 p53 −/− cell line. Viability assays in the HCT116 (E) and LoVo (F) cell lines. Genetic over-expression of HO-1 decreased cell number in both cell lines ($^{**}p = 0.0017$, $^{**}p = 0.0023$). G. Protein lysates of transiently transfected HCT116 cells were electrophoresed and blotted against HO-1, pEGFP, p53, p21, p27, Bax, PKC βI, and PKCβII. Actin was used as loading control.

this, we have also demonstrated that the expression of HO-1 in tumors is associated with increased overall survival of patients with CRC. These results are in agreement with previous studies of HO-1 expression in CRC where a correlation between HO-1 positivity and a better long term survival was found (Becker et al., 2007). However, they are contrary to the majority of the tumor types analyzed in which HO-1 has been positively associated with tumor progression (Was et al., 2006). The exceptions to this pro-tumoral role for HO-1 have been the findings of Becker and col in CRC already mentioned (Becker et al., 2007) and the observations in tongue squamous cell carcinomas (Yanagawa et al., 2004), mammary tumors (Hill et al., 2005 and unpublished observations from our laboratory) and prostate cancer (Gueron et al., 2009).

In order to further demonstrate that HO-1 expression increases with tumor progression, we also evaluated its expression in a CRC animal model. HO-1 up-regulation as well as an increase in the incidence of nuclear localization were observed during tumor progression. Furthermore, HO-1 nuclear expression was associated with less differentiated, more aggressive tumors in the animal model. This nuclear expression was also observed in human samples. These results are in agreement with the observations of Yin et al. (2014) where an increase in nuclear HO-1 was observed with advanced tumor stages. Nuclear localization was also reported by our group in human samples and in an animal model of squamous cell carcinoma (Gandini et al., 2012) and also agree with previous reports demonstrating an increase in HO-1 nuclear staining when oral epithelial dysplasias progress from moderate to severe (Lee et al., 2008). Similar results were also obtained in prostate cancer in which HO-1 nuclear expression was found to be lower in adjacent non-malignant tissues than in prostate carcinoma ones (Sacca et al., 2007). Additionally, in support of a role of nuclear HO-1 in tumor progression, a recent report demonstrated that cigarette smoke induces nuclear translocation of HO-1, and this localization promotes vascular endothelial growth factor secretion, which favors prostate tumor progression (Birrane et al., 2013). Although this novel nuclear localization of HO-1 has been demonstrated in some tissues, its significant role has not been completely addressed. In this regard, it was postulated that the nuclear form of HO-1 may up-regulate genes that promote cytoprotection against oxidative stress (Lin et al., 2007). There is evidence showing that nuclear HO-1 could have a physiological role independent of its enzymatic activity. For example, gene transfection of the activity-lacking mutant HO-1 protects cells against oxidative stress (Busserolles et al., 2006). As the HO-1 structure does not show DNA-binding motifs it seems that this protein is not a typical transcription factor (Lin et al., 2007). However, HO-1, acting as a transcriptional co-regulator protein, may be able to modulate transcription factors, nuclear localization being thus necessary for these effects, and this explains its presence in the nuclear compartment.

As already stated, most of the literature shows a pro-tumoral role of HO-1 (Was et al., 2006) with a few exceptions already mentioned. Since we observed an association of HO-1 with longer patients' survival time, we hypothesized that HO-1 expression in CRC cells could inhibit cellular survival. Our results demonstrate that HO-1 activation decreases cell survival through cell cycle arrest and induction of apoptosis. Importantly,

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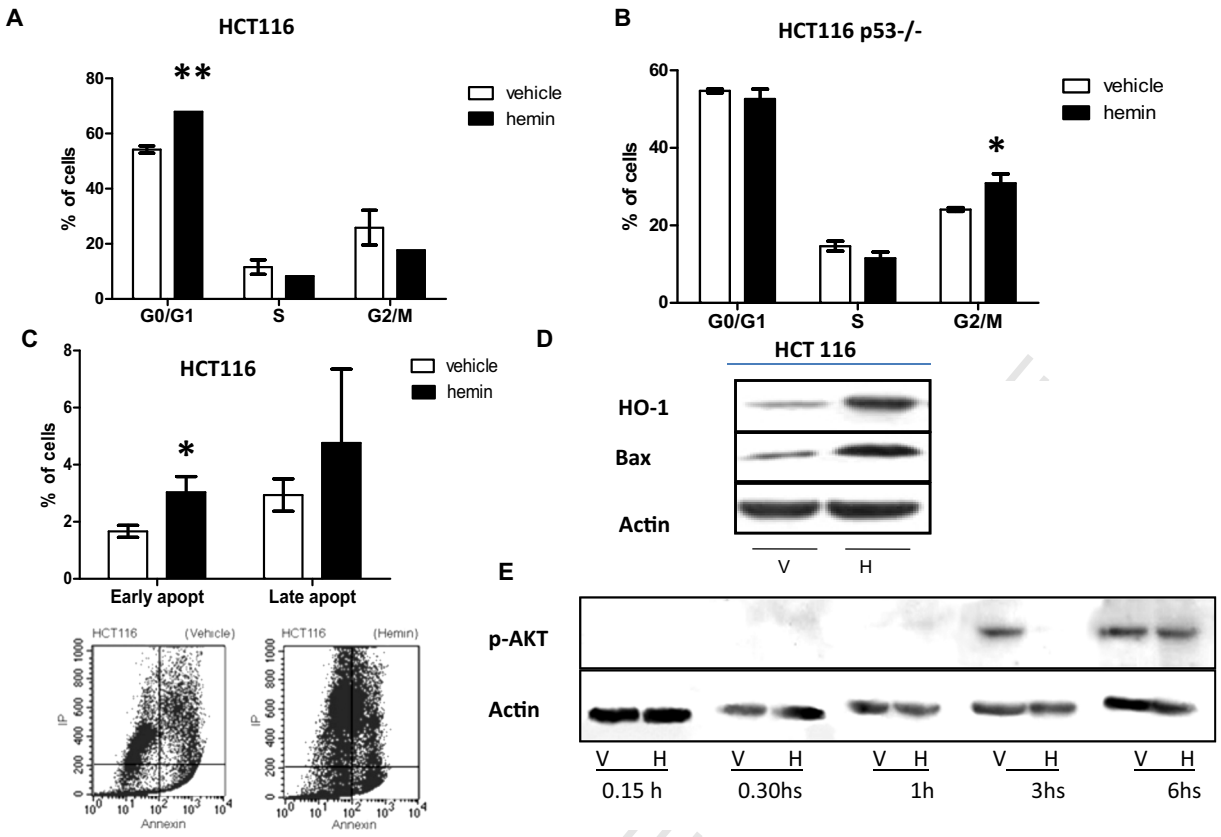

Fig. 6. HO-1 produces cell cycle arrest in G_0/G_1 and early apoptosis in HCT116 cells and modulates the expression of the pro-apoptotic protein Bax. A and B. Distribution of the phases of the cell cycle by determining the DNA content carried out by flow cytometry using IP staining. The histograms show the percentage of cells from each cell line, HCT116 (A) and HCT116 p53 −/− (B), located in different cell cycle phases after treatment with hemin and vehicle (100 μM). Cell arrest was observed in G_0/G_1 phase in those cells that have wild type p53 ($^{**}p = 0.004$) and G2/M in cells lacking p53 ($^{*}p < 0.05$). C. Detection of apoptosis in HCT116 cells. The cells were analyzed by flow cytometry using Annexin-V staining. Percentage of cells in early apoptosis ($^{*}p = 0.01$) and late apoptosis ($p > 0.17$) after treatment with hemin (100 μM) and vehicle. D. HCT116 cells were treated with vehicle (V) and hemin (H, 100 μM) for 24 h and HO-1 and Bax expression determined. Actin was used as loading control. E. The HCT116 cells were subjected to a 6-hour pretreatment with vehicle (V) and hemin (H); then they were maintained for 16 h in serum-free medium and subsequently treated for 15 min, 30 min, 1 h, 3 h and 6 h with vehicle (V) or hemin (H, 100 μM) in medium containing serum. The expression of phosphorylated Akt was analyzed by western blot. The results were normalized with actin.

p53 tumor suppressor protein is necessary for these effects, since HO-1 reduction in cell survival is blunted in the HCT116 cell line lacking p53 and in the HT29 which bears a mutated p53. On the other hand, previous work from other laboratories done with the p53-mutated Caco-2 cell line showed an anti-apoptotic activity for HO-1 (Busserolles et al., 2006). Altogether, these results support the hypothesis of HO-1 decreasing survival in CRC through wild type p53 up-regulation. p53 tumor suppressor gene is mutated in over 50% of human tumors and plays an important role in the response to genotoxic stress and hypoxia. The contradictory responses to HO-1 activation observed in different tumor cells might be explained by the different status of p53 that they carry. However, the relationship between HO-1 and p53 is not yet clear as there are some reports showing that p53 induces HO-1, and other ones demonstrating that HO-1 is upstream of p53. For example in the lymphoid organs of γ-irradiated mice, HO-1 was demonstrated to be a direct p53 target gene (Meiller et al., 2007). Nam et al. also demonstrated a role for p53 in promoting cellular survival through the activation of HO-1 (Nam and Sabapathy, 2011). On the other hand, Lee et al. (2008) have demonstrated that HO activity is involved in the regulation of p53 expression in a human retinal pigment epithelial cell line and Kim et al. (2014) showed that HO-1 is necessary for the up-regulation of p53 induced by 15d-PGJ2 in breast cancer cells. Importantly, in non-small cell lung carcinoma HO-1 up-regulated p53 and this was accompanied by a reduction in proliferation, migration and angiogenic potential (Skrzypek et al., 2013). Additional investigations should be performed in order to further establish wild-type p53 as responsible for HO-1 effects on cellular survival in CRC.

Part of the observed effects upon HO-1 activation may be secondary to modulation of Akt and PKC β pathways. The role of PKC β is controversial and varies in different cell lines. According to the results obtained by Choi et al. (1990) PKC βI is associated with reduced tumorigenicity, whereas activation of PKC βII isoform plays a direct role in increasing colorectal cancer cell proliferation (Sauma et al., 1996). The role of Akt pathway in CRC is very well understood and modulates both cell survival and migration processes (Agarwal et al., 2013). Finally, we demonstrated that HO-1 activation reduces cell migration. This action could involve modulation of the matrix metalloproteinases (MMP)'s levels specially the gelatinases MMP-2 and MMP-9 that have important roles in tumor invasion, metastasis and angiogenesis in colorectal cancer (Tutton et al., 2003). In relation to this, there is evidence that indicates that HO-1 reduces the MMP-9 levels (Gueron et al., 2009).

In conclusion, we provide evidence that HO-1 is up-regulated during CRC progression and this overexpression is associated with an increase in the overall survival time of patients. We also provide evidence that demonstrates that the effects of HO-1 in CRC involve a decrease in cellular migration and survival and that the p53 tumor suppressor protein is necessary for this effect. Altogether these results point to an antitumoral role of HO-1 in wild-type-p53-bearing CRC.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yexmp.2014.09.012.

Conflict of interest statement

The authors disclose no potential conflicts of interest.

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References

Agarwal, E., Brattain, M.G., Chowdhury, S., 2013. Cell survival and metastasis regulation by Akt signaling in colorectal cancer. Cell. Signal. 25, 1711–1719. http://dx.doi.org/10.1016/j.cellsig.2013.03.025.

Barton, S.G., Rampton, D.S., Winrow, V.R., Domizio, P., Feakins, R.M., 2003. Expression of heat shock protein 32 (hemoxygenase-1) in the normal and inflamed human stomach and colon: an immunohistochemical study. Cell Stress Chaperones 8, 329–334.

Becker, J.C., Fukui, H., Imai, Y., Sekikawa, A., Kimura, T., Yamagishi, H., Yoshitake, N., Pohle, T., Domschke, W., Fujimori, T., 2007. Colonic expression of heme oxygenase-1 is associated with a better long-term survival in patients with colorectal cancer. Scand. J. Gastroenterol. 42, 852–858. http://dx.doi.org/10.1080/00365520701192383.

Berberat, P.O., Dambrauskas, Z., Gulbinas, A., Giese, T., Giese, N., Künzli, B., Autschbach, F., Meuer, S., Büchler, M.W., Friess, H., 2005. Inhibition of heme oxygenase-1 increases responsiveness of pancreatic cancer cells to anticancer treatment. Clin. Cancer Res. 11, 3790–3798. http://dx.doi.org/10.1158/1078-0432.CCR-04-2159.

Birrane, G., Li, H., Yang, S., Tachado, S.D., Sen, G.S., 2013. Cigarette smoke induces nuclear translocation of heme oxygenase 1 (HO-1) in prostate cancer cells: nuclear HO-1 promotes vascular endothelial growth factor secretion. Int. J. Oncol. 42, 1919–1928. http://dx.doi.org/10.3892/ijo.2013.1910.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. http://dx.doi.org/10.1016/0003-2697(76)90527-3.

Busserolles, J., Megías, J., Terencio, M.C., Alcaraz, M.J., 2006. Heme oxygenase-1 inhibits apoptosis in Caco-2 cells via activation of Akt pathway. Int. J. Biochem. Cell Biol. 38 (9), 1510–1517.

Chipuk, J.E., Kuwana, T., Bouchier-Hayes, L., Droin, N.M., Newmeyer, D.D., Schuler, M., Green, D.R., 2004. Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. Science 303, 1010–1014. http://dx.doi.org/10.1126/science.1092734.

Choi, P.M., Tchou-Wong, K.M., Weinstein, I.B., 1990. Overexpression of PKC in HT29 colon cancer cells causes growth inhibition and tumor suppression. Mol. Cell. Biol. 10, 4650–4657. http://dx.doi.org/10.1089/ars.2013.5184.

Compton, C.C., Greene, F.L., 2004. The staging of colorectal cancer: 2004 and beyond. CA Cancer J. Clin. 54, 295–308. http://dx.doi.org/10.3322/canjclin.54.6.295.

Degese, M.S., Mendizabal, J.E., Gandini, N.A., Gutkind, J.S., Molinolo, A., Hewitt, S.M., Curino, A.C., Coso, O.A., Facchinetti, M.M., 2012. Expression of heme oxygenase-1 in non-small cell lung cancer (NSCLC) and its correlation with clinical data. Lung Cancer 77, 168–175. http://dx.doi.org/10.1016/j.

Deininger, M.H., Meyermann, R., Trautmann, K., Duffner, F., Grote, E.H., Wickboldt, J., Schluesener, H.J., 2000. Heme oxygenase (HO)-1 expressing macrophages/microglial cells accumulate in perinecrotic areas of rat and human gliomas. Brain Res. 882, 1–8. http://dx.doi.org/10.1016/S0006-8993(00)02594-4.

Doi, K., Akaike, T., Fujii, S., Tanaka, S., Ikebe, N., Beppu, T., Shibahara, S., Ogawa, M., Maeda, H., 1999. Induction of haem oxygenase-1 nitric oxide and ischaemia in experimental solid tumours and implications for tumour growth. Br. J. Cancer 80, 1945–1954. http://dx.doi.org/10.1038/sj.bjc.6690624.

Facchinetti, M.M., Gandini, N.A., Fermento, M.E., Sterin-Speziale, N.B., Ji, Y., Patel, V., Gutkind, J.S., Rivadulla, M.G., Curino, A.C., 2010. The expression of sphingosine kinase-1 in head and neck carcinoma. Cells Tissues Organs 192, 314–324. http://dx.doi.org/10.1159/000318173.

Ferlay, J., Shin, H.R., Bray, F., Forman, D., Mathers, C., Parkin, D.M., 2010. GLOBOCAN 2008 v2.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 10 [Internet]. International Agency for Research on Cancer, Lyon, France (2010. Available from: http://globocan.iarc.fr, accessed on 20/11/2013).

Gandini, N.A., Fermento, M.A., Salomón, D., Blasco, J., Patel, V., Gutkind, J.S., Molinolo, A.A., Facchinetti, M.M., Curino, A.C., 2012. Nuclear localization of heme oxygenase-1 is associated with tumor progression of head and neck squamous cell carcinomas. Exp. Mol. Pathol. 93, 237–245. http://dx.doi.org/10.1016/j.yexmp.2012.05.001.

Gandini, N.A., Fermento, M.E., Salomón, D.G., Obiol, D.J., Andrés, N.C., Zenklusen, J.C., Arevalo, J., Blasco, J., López Romero, A., Facchinetti, M.M., Curino, A.C., 2014. Heme oxygenase-1 expression in human gliomas and its correlation with poor prognosis in patients with astrocytoma. Tumor Biol. 35 (3), 2803–2815. http://dx.doi.org/10.1007/s13277-013-1373-z.

Giriş, M., Erbil, Y., Oztezcan, S., Olgaç, V., Barbaros, U., Deveci, U., Kirgiz, B., Uysal, M., Toker, G.A., 2006. The effect of heme oxygenase-1 induction by glutamine on radiation-induced intestinal damage: the effect of heme oxygenase-1 on radiation enteritis. Am. J. Surg. 191, 503–509. http://dx.doi.org/10.1016/j.amjsurg.2005.11.004.

Goodman, A.I., Choudhury, M., da Silva, J.L., Schwartzman, M.L., Abraham, N.G., 1997. Overexpression of the heme oxygenase gene in renal cell carcinoma. Proc. Soc. Exp. Biol. Med. 214, 54–61. http://dx.doi.org/10.3181/00379727-214-44069.

Gueron, G., De Siervi, A., Ferrando, M., Salierno, M., De Luca, P., Elguero, B., Meiss, R., Navone, N., Vazquez, E.S., 2009. Critical role of endogenous heme oxygenase 1 as a tuner of the invasive potential of prostate cancer cells. Mol. Cancer Res. 7, 1745–1755. http://dx.doi.org/10.1158/1541-7786.

Guo, J.S., Cho, C.H., Wang, J.Y., Koo, M.W., 2002. Expression and immunolocalisation of heat shock proteins in the healing of gastric ulcers in rats. Scand. J. Gastroenterol. 37, 17–22. http://dx.doi.org/10.1080/003655202753387293.

Hara, E., Takahashi, K., Tominaga, T., Kumabe, T., Kayama, T., Suzuki, H., Fujita, H., Yoshimoto, T., Shirato, K., Shibahara, S., 1996. Expression of heme oxygenase and inducible nitric oxide synthase mRNA in human brain tumours. Biochem. Biophys. Res. Commun. 224, 153–158. http://dx.doi.org/10.1006/bbrc.1996.0999.

Hill, M., Pereira, V., Chauveau, C., Zagani, R., Remy, S., Tesson, L., Mazal, D., Ubillos, L., Brion, R., Asghar, K., Mashreghi, M.F., Kotsch, K., Moffett, J., Doebis, C., Seifert, M., Boczkowski, J., Osinaga, E., Anegon, I., 2005. Heme oxygenase-1 inhibits rat and human breast cancer cell proliferation: mutual cross inhibition with indoleamine 2,3-dioxygenase. FASEB J. 19, 1957–1968. http://dx.doi.org/10.1096/fj.05-3875com.

Jozkowicz, A., Was, H., Dulak, J., 2007. Heme oxygenase-1 in tumors: is it a false friend? Antioxid. Redox Signal. 9, 2099–2117. http://dx.doi.org/10.1089/ars.2007.1659.

Kang, K.A., Maeng, Y.H., Zhang, R., Yang, Y.R., Piao, M.J., Kim, K.C., Kim, G.Y., Kim, Y.R., Koh, Y.S., Kang, H.K., Hyun, C.L., Chang, W.Y., Hyun, J.W., 2012. Involvement of heme oxygenase-1 in Korean colon cancer. Tumour Biol. 33, 1031–1038. http://dx.doi.org/10.1007/s13277-012-0336-0.

Kim, D.H., Song, N.Y., Kim, E.H., Na, H.K., Joe, Y., Chung, H.T., Surh, Y., 2014. J5-deoxy-Δ(12,14)-prostaglandin J2 induces p53 expression through Nrf2-mediated upregulation of heme oxygenase-1 in human breast cancer cells. Free Radic. Res. 48 (9), 1018–1027. http://dx.doi.org/10.3109/10715762.2014.897343.

La, P., Fernando, A.P., Wang, Z., Salahudeen, A., Yang, G., Lin, Q., Wright, C.J., Dennery, P.A., 2009. Zinc protoporphyrin regulates cyclin D1 expression independent of heme oxygenase inhibition. J. Biol. Chem. 284, 36302–36311. http://dx.doi.org/10.1074/jbc.M109.03164.

Lee, S.Y., Jo, H.J., Kim, K.M., Song, J.D., Chung, H.T., Park, Y.C., 2008. Concurrent expression of heme oxygenase-1 and p53 in human retinal pigment epithelial cell line. Biochem. Biophys. Res. Commun. 365, 870–874. http://dx.doi.org/10.1016/j.bbrc.2007.11.0.

Liefers, G.J., Tollenaar, R.A., 2002. Cancer genetics and their application to individualized medicine. Eur. J. Cancer 38, 872–879. http://dx.doi.org/10.1016/S0959-8049(02)00055-2.

Lin, Q., Weis, S., Yang, G., Weng, Y.H., Helston, R., Rish, K., Smith, A., Bordner, J., Polte, T., Gaunitz, F., Dennery, P.A., 2007. Heme oxygenase-1 protein localizes to the nucleus and activates transcription factors important in oxidative stress. J. Biol. Chem. 282, 20621–20633. http://dx.doi.org/10.1074/jbc.M607954200.

Maines, M.D., Gibbs, P.E., 2005. 30 some years of heme oxygenase: from a "molecular wrecking ball" to a "mesmerizing" trigger of cellular events. Biochem. Biophys. Res. Commun. 338, 568–577. http://dx.doi.org/10.1016/j.bbrc.2005.08.121.

Mayerhofer, M., Florian, S., Krauth, M.T., Aichberger, K.J., Bilban, M., Marculescu, R., Printz, D., Fritsch, G., Wagner, O., Selzer, E., Sperr, W.R., Valent, P., Sillaber, C., 2004. Identification of heme oxygenase-1 as a novel BCR/ABL-dependent survival factor in chronic myeloid leukemia. Cancer Res. 64, 3148–3154. http://dx.doi.org/10.1158/0008-5472.CAN-03-120.

McAllister, S.C., Hansen, S.G., Ruhl, R.A., Raggo, C.M., DeFilippis, V.R., Greenspan, D., Früh, K., Moses, A.V., 2004. Kaposi sarcoma-associated herpesvirus (KSHV) induces heme oxygenase-1 expression and activity in KSHV-infected endothelial cells. Blood 103, 3465–3473. http://dx.doi.org/10.1182/blood-2003-08-2781.

Meiller, A., Alvarez, S., Drané, P., Lallemand, C., Blanchard, B., Tovey, M., May, E., 2007. p53-dependent stimulation of redox-related genes in the lymphoid organs of gamma-irradiated-mice identification of Haeme-oxygenase 1 as a direct p53 target gene. Nucleic Acids Res. 35, 6924-6234. http://dx.doi.org/10.1093/nar/gkm824.

Nam, S.Y., Sabapathy, K., 2011. p53 promotes cellular survival in a context-dependent manner by directly inducing the expression of haeme-oxygenase-1. Oncogene 30, 4476–4486. http://dx.doi.org/10.1038/onc.2011.150.

Nissan, A., Guillem, J.G., Paty, P.B., Wong, W.D., Cohen, A.M., 1999. Signet-ring cell carcinoma of the colon and rectum: a matched control study. Dis. Colon Rectum 42, 1176–1180.

Oliver, M., Hollstein, M., Hainaut, P., 2010. TP53 mutations in human cancers: origins, consequences, and clinical use. Cold Spring Harb. Perspect. Biol. 2, a001008. http://dx.doi.org/10.1101/cshperspect.a001008.

Paul, G., Bataille, F., Obermeier, F., Bock, J., Klebl, F., Strauch, U., Lochbaum, D., Rümmele, P., Farkas, S., Schölmerich, J., Fleck, M., Rogler, G., Herfarth, H., 2005. Analysis of intestinal haem-oxygenase-1 (HO-1) in clinical and experimental colitis. Clin. Exp. Immunol. 140, 547–555. http://dx.doi.org/10.1111/j.1365-2249.2005.02775.x.

Petit, V., Boyer, B., Lentz, D., Turner, C.E., Thiery, J.P., Valles, A.M., 2000. Phosphorylation of tyrosine residues 31 and 118 on paxillin regulates cell migration through an association with CRK in NBT-II cells. J. Cell Biol. 148, 957–970.

Sacca, P., Meiss, R., Casas, G., Mazza, O., Calvo, J.C., Navone, N., Vazquez, E., 2007. Nuclear translocation of haeme oxygenase-1 is associated to prostate cancer. Br. J. Cancer 97, 1683–1689. http://dx.doi.org/10.1038/sj.bjc.6604081.

Sauma, S., Yan, Z., Ohno, S., Friedman, E., 1996. Protein kinase Cb1 and protein kinase Cb2 activate p57 mitogen-activated protein kinase and block differentiation in colon carcinoma cells. Cell Growth Differ. 7, 587–594.

Schacter, B.A., Kurz, P., 1982. Alterations in hepatic and splenic microsomal 28 electron transport system components, drug metabolism, heme oxygenase activity, and cytochrome P-450 turnover in Murphy–Sturm lymphosarcoma-bearing rats. Cancer Res. 42, 3557–3564.

Skrzypek, K., Tertil, M., Golda, S., Ciesla, M., Weglarczyk, K., Collet, G., Guichard, A., Kozakowska, M., Boczkowski, J., Was, H., Gil, T., Kuzdzal, J., Muchova, L., Vitek, L., Loboda, A., Jozkowicz, A., Kieda, C., Dulak, J., 2013. Interplay between heme oxygenase-1 and miR-378 affects non-small cell lung carcinoma growth, vascularization, and metastasis. Antioxid. Redox Signal. 19, 644–660. http://dx.doi.org/10.1089/ars.2013.5184.

Takagi, T., Naito, Y., Mizushima, K., Nukigi, Y., Okada, H., Suzuki, T., Hirata, I., Omatsu, T., Okayama, T., Handa, O., Kokura, S., Ichikawa, H., Yoshikawa, T., 2008. Increased

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intestinal expression of heme oxygenase-1 and its localization in patients with ulcerative colitis. J. Gastroenterol. Hepatol. 23, 229–233. http://dx.doi.org/10.1111/j.1440-1746.2008.05443.x.

Torisu-Itakura, H., Furue, M., Kuwano, M., Ono, M., 2000. Co-expression of thymidine phosphorylase and heme oxygenase-1 in macrophages in human malignant vertical growth melanomas. Jpn. J. Cancer Res. 91, 906–910.

Tutton, M.G., George, M.L., Eccles, S.A., Burton, S., Swift, R.I., Abulafi, A.M., 2003. Use of plasma mmp-2 and mmp-9 levels as a surrogate for Tumor expression in colorectal cancer patients. Int. J. Cancer 107, 541–550. http://dx.doi.org/10.1002/ijc.11436.

Wang, W.P., Guo, X., Koo, M.W., Wong, B.C., Lam, S.K., Ye, Y., Cho, C.H., 2001. Protective role of heme oxygenase-1 in trinitrobenzene sulphonic acid-induced colitis in rats. Am. J. Physiol. Gastrointest. Liver Physiol. 281, G586–G594.

Was, H., Cichon, T., Smolarczyk, R., Rudnicka, D., Stopa, M., Chevalier, C., Leger, J.J., Lackowska, B., Grochot, A., Bojkowska, K., Ratajska, A., Kieda, C., Szala, S., Dulak, J., Jozkowicz, A., 2006. Overexpression of heme oxygenase-1 in murine melanoma: increased proliferation and viability of tumor cells, decreased survival of mice. Am. J. Pathol. 169, 2181–2198. http://dx.doi.org/10.2353/ajpath.2006.051365.

Yanagawa, T., Omura, K., Harada, H., Nakaso, K., Iwasa, S., Koyama, Y., Onizawa, K., Yusa, H., Yoshida, H., 2004. Heme oxygenase-1 expression predicts cervical lymph node metastasis of tongue squamous cell carcinomas. Oral Oncol. 40, 21–27. http://dx.doi.org/10.1016/S1368-8375(03)00128-3.

Yin, H., Fang, J., Liao, L., Maeda, H., Su, Q., 2014. Upregulation of heme oxygenase-1 in colorectal cancer patients with increased circulation carbon monoxide levels, potentially affects chemotherapeutic sensitivity. BMC Cancer 14, 436. http://dx.doi.org/10.1186/1471-2407-14-436.

Zhu, M.H., Sung, I.K., Zheng, H., Sung, T.S., Britton, F.C., O'Driscoll, K., Koh, S.D., Sanders, K. M., 2011. Muscarinic activation of Ca^{2+}-activated Cl^{-} current in interstitial cells of Cajal. J. Physiol. 589, 4565–4582. http://dx.doi.org/10.1113/jphysiol.2011.211094.

Please cite this article as: Andrés, N.C., et al., Heme oxygenase-1 has antitumoral effects in colorectal cancer: Involvement of p53, Exp. Mol. Pathol. (2014), http://dx.doi.org/10.1016/j.yexmp.2014.09.012