

# Haptoglobin expression in human colorectal cancer

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**Summary.** Aims and experimental design. The acute-phase protein haptoglobin (Hp) has been recently detected in colorectal cancer (CRC) tissue, where its expression correlates with metastasis. Recently, we identified Hp as a CDw75 antigen-expressing protein in colorectal tissue. To deepen the knowledge of this protein in CRC, we studied the expression of Hp in healthy and tumour tissue specimens from 62 CRC patients by immunohistochemistry and Western blotting, as well as in the Caco-2 and HT-29 CRC cell lines by quantitative PCR, immunofluorescence microscopy and flow cytometry. Results and discussion. Hp immunopositive staining was absent in the 18 healthy colorectal specimens analysed, whereas it was observed in 24% (15/62) of the tumour specimens as cytoplasmic granules within cancer cells. Furthermore, Hp expression in CRC was associated with Dukes' stage and the presence of metastasis in our population of study. *In vitro* cultured Caco-2 and HT-29 cells expressed mRNA for Hp and the protein was detected at the cell surface. Conclusions. This study confirms the expression of Hp in CRC, both *in vivo* and *in vitro*, and provides further evidence of its association with disease progression and metastasis.

**Key words:** Haptoglobin, Human colorectal cancer tissue, Metastasis, Caco-2, HT-29

## Introduction

Haptoglobin (Hp) is an acute-phase protein whose levels increase in inflammatory conditions such as trauma, wounds, infarction, infection and cancer (Wang et al., 2001). Most Hp is synthesised in the liver, but significant amounts are also expressed by the oral (Lee et al., 2010), lung (Abdullah et al., 2009) and colorectal epithelia (Uhlén et al., 2005). Apart from the binding and transport of the haemoglobin released after erythrocyte senescence or turnover (Ascenzi et al., 2005), Hp also possesses immunosuppressive properties (Israël et al., 1981) such as the inhibition of mast cell (El-Ghmati et al., 2002) and T/B lymphocyte proliferation (Arredouani et al., 2003) or the inhibition of nitric oxide-mediated vasodilation (Edwards et al., 1986). In fact, Hp expression increases in response to pro-inflammatory cytokines such as tumour necrosis factor (TNF)- $\alpha$  and interleukins IL-1 and IL-6 (Baumann et al., 1989). Additionally, Hp promotes angiogenesis (Cid et al., 1993), cell migration (de Kleijn et al., 2002) and has anti-inflammatory properties through the inhibition of prostaglandin synthesis (Langlois and Delangue, 1996).

We have recently identified Hp as a glycoprotein that probably bears the CDw75 glycotope (Neu5Ac $\alpha$ (2,6)Gal $\beta$ (1,4)GlcNAc) in the tumour tissue from colorectal cancer (CRC) patients (Mariño-Crespo et al., 2018). Initially described as a differentiation antigen of B lymphocytes (Dorken et al., 1989), more recent evidence has shown that CDw75 is overexpressed in CRC tissue (Elpek et al., 2002; Costa-Nogueira et al., 2009), where it is associated with disease progression (Villar-Portela et al., 2011).

Worldwide, neoplasias of colon and rectum represent the third most common type of cancer (new diagnoses) and the fourth leading cause of tumour-associated death (GBDCC, 2015). Consequently, there is great interest in identifying new prognostic biomarkers to improve clinical management of CRC patients. In this regard, the increase of serum Hp concentration in CRC patients (Bresalier et al., 2004; Sun et al., 2016) has been recently proposed, together with CEA and CA19.9, as biomarker for the early detection of hepatic metastasis (Sun et al., 2016), and high levels of the protein have been linked to a greater risk of CRC-associated death (Ghuman et al., 2017). In addition, serum Hp from CRC patients displays several aberrant glycosylation traits, such as more abundant N-acetyl-lactosamination (Bresalier et al., 2004) and fucosylation (Park et al., 2012; Takeda et al., 2012). Moreover, histological studies have previously shown Hp expression restricted to colorectal tumour tissue (Harvey et al., 2009), and associated with tumour infiltration, lymph node involvement and metastasis, where it appears to be a positive predictor of hepatic metastasis and lower survival rate of CRC patients (Sun et al., 2012).

All the evidence sustains the importance of Hp expression in CRC carcinogenesis and tumour progression, making the research on this protein a matter of interest. Therefore, in order to better characterize the expression of Hp in CRC, we have 1) evaluated Hp mRNA expression by RT-qPCR and screened the subcellular localization of the protein by flow cytometry (FC) and immunofluorescence microscopy (IF) in Caco-2 and HT-29 CRC cell lines; 2) examined its expression in healthy and tumour tissue from CRC patients by immunohistochemistry (IHC) and Western blotting (WB); and 3) analysed its association with clinicopathological features. The results obtained confirm that epithelial cells from tumour colorectal mucosa express Hp and that this expression is associated with some tumour clinicopathological features with prognostic potential for CRC patients.

## **Materials and methods**

### *Ethics and consent*

The study received the approval from the Ethical Committee of Clinical Research of Galicia (Health Department, Xunta de Galicia, Spain) on May 27, 2010 (expt. no. 2007/177). In accordance with its ethical statements, patients accepted and signed the corresponding informed consent prior to surgery. The numerical identifiers of the biopsies, as well as the clinicopathological features of the patients, were always managed anonymously by the authors.

### *Patients and tissue specimens*

A total of 62 CRC patients diagnosed at the University Hospital Complex of Ourense (CHUO, Ourense, Spain) between 2004 and 2010, were randomly

selected for this study. Fresh specimens of tumour and healthy tissue (taken at a distance of at least 10 cm from the tumour) were obtained during surgical resection of the adenocarcinomas, washed with ice-cold saline buffer, and kept at  $-80^{\circ}\text{C}$  until use. Additionally, the specimens for IHC analysis were fixed in 10% PBS-buffered formalin and embedded in paraffin following the standard protocol. Tumour staging was performed by expert pathologists according to Dukes' staging (Dukes, 1932) and TNM classification (Union of International Cancer Control, 2009).

### *Cell culture*

The human CRC cell line Caco-2 (ECACC 86010202) and the human hepatocyte carcinoma cell line, HepG2 (ECACC 85011430) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum, 100 IU penicillin, 100  $\mu\text{g}/\text{mL}$  streptomycin and 2 mM L-glutamine (Sigma-Aldrich). HT-29 human CRC cells (ECACC 91072201) were cultured in McCoy's 5A medium supplemented as for DMEM. All cells were cultured at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$ . For cytokine IL-6 stimulation studies, cells were seeded at  $2 \times 10^6$  cells in a  $25 \text{ cm}^2$  flask and incubated for 24 h. Cells were then washed with PBS, cultured for 1 h with serum-free medium and then stimulated with 100 ng/mL recombinant IL-6 (Perprotech, UK) for 3 h at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$ . Controls received medium alone.

### *Immunohistochemistry*

The IHC study included 80 colorectal tissue specimens (18 healthy and 62 tumour specimens) from 62 CRC patients. Moreover, several hepatic samples were included as positive controls for Hp expression. The  $3\text{-}\mu\text{m}$  histological sections from paraffin-embedded tissue blocks were deparaffinised and rehydrated previous to antigen retrieval in boiling 10 mM citric acid (pH 6.0) for 15 min. Endogenous peroxidase activity was blocked with a  $\text{H}_2\text{O}_2$  solution (EnVision™ FLEX Peroxidase-Blocking Reagent, Dako). To avoid non-specific binding, sections were further incubated with 10% goat serum for 20 min prior to the addition of the primary antibody (monoclonal mouse anti-human Hp, 1:50, ab13429, Abcam) for 1 h in moist chamber. Sections were then incubated with a HRP-conjugated secondary antibody (goat anti-mouse labelled polymer, HRP, EnVision™ Detection System, Dako) for 30 min in moist chamber. Next, slides were treated with 3,3'-diaminobenzidine reagent (DAB, EnVision™ Detection System, Dako) and counterstained with Papanicolaou's haematoxylin. Finally, the tissue sections were dehydrated and mounted in DPX medium. Negative controls were serial sections from the same histological blocks used for IHC testing and assayed in the same experimental conditions, except by using PBS instead of the primary antibody.

The Hp staining was evaluated by the Pathology

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Service of CHUO, according to the following semi-quantitative scale of Hp-positive epithelial cells: 0 = no staining, 1 = weak staining intensity (less than 10% of cells stained), 2 = moderate staining intensity (10-50% of cells stained), 3 = strong staining intensity (more than 50% of cells positive for Hp).

### Western blotting

WB was performed on paired healthy and tumour fresh-frozen tissue specimens from five CRC patients previously analysed by IHC. Enriched fractions of total cell membranes and cytosolic proteins were obtained (Mariño-Crespo et al., 2018) and their protein concentration determined by the bicinchoninic acid protein assay (Sigma-Aldrich).

Twenty  $\mu\text{g}$  of protein from the subcellular fractions, along with diluted serum samples from healthy donors (obtained from the Galician Transfusion Centre) used as Hp-positive controls, were separated in 10% SDS-PAGE gels, then transferred to PVDF membranes (Hybond P 0.45, Amersham) for 80 min at 120 V, blocked with 5% non-fat dry milk in PBS for 1 h, and immunoblotted with an anti-Hp monoclonal (1:500, ab13429, Abcam) for 1 h and a HRP-conjugated secondary antibody (goat anti-mouse IgG, 0.03  $\mu\text{g}/\text{mL}$ , ab97040, Abcam) for 1 h. Negative controls lacked the primary anti-Hp antibody. Finally, membranes were developed with Clarity™ Western ECL Substrate (Bio-Rad). The protein bands were visualised in a ChemiDoc™ XRS+ system (Bio-Rad). Quantification was performed by a normalization process that uses the Coomassie staining of the membranes as a loading control (Welinder and Ekblad, 2011).

### Flow cytometry

Confluent Caco-2 and HT-29 cells were detached from culture flasks with trypsin-EDTA or, alternatively, with a non-enzymatic cell dissociation solution. Then,  $5 \times 10^5$  cells/assay were incubated for 45 min at 4°C with either anti-Hp monoclonal antibody (1:40, ab13429, Abcam), a cell surface staining control (anti-HLA Class I, 13  $\mu\text{g}/\text{mL}$ , 14-9983, eBioscience) or an isotype control antibody (10  $\mu\text{g}/\text{mL}$ , MAB002, R&D Systems). Cells were subsequently incubated in the dark with an Alexa Fluor® 488-labelled secondary antibody (20  $\mu\text{g}/\text{mL}$ , A11001, Life Technologies) for 30 min at 4°C.

Five  $\mu\text{M}$  TO-PRO®-3 (Life Technologies) were added in some assays to allow gating of viable cells. The FC analysis consisted of the acquisition of data from  $10^4$  events with a FACSCalibur (BD Biosciences) device. The specific fluorescence of Hp was calculated by subtracting the isotype control signal from the total Hp signal.

### Immunofluorescence microscopy

Caco-2 and HT-29 cells were cultured overnight on glass slides at a density of  $2.5 \times 10^4$  cells/well. Cells were either fixed in 2% paraformaldehyde or permeabilized in

freezing cold methanol. Non-specific binding was reduced by incubating the samples with 5% goat serum in PBS. Next, slides were incubated for 1 h at 4°C with an anti-Hp primary monoclonal antibody (1:40, ab13429, Abcam), a cell surface staining control (anti-HLA, 5  $\mu\text{g}/\text{mL}$ , 14-9983, eBioscience), a permeabilization control (anti- $\beta$ -actin, 2  $\mu\text{g}/\text{mL}$ , A1978, Sigma-Aldrich) or without antibody (negative controls). Samples were then incubated in the dark with an Alexa Fluor® 488-conjugated secondary antibody (20  $\mu\text{g}/\text{mL}$ , A11001, Life Technologies) for 45 min at 4°C. Cell nuclei were counterstained with 5  $\mu\text{g}/\text{mL}$  DAPI (Life Technologies) and finally the slides were mounted in ProLong Gold antifade mounting medium (Life Technologies).

### Reverse transcription and quantitative polymerase chain reaction (RT-qPCR)

Total RNA from HT-29, Caco-2 and HepG2 cells cultured as previously indicated (*Cell culture*) was isolated using Monarch® Miniprep kit (New England Biolabs Inc.) and 1  $\mu\text{g}$  reverse transcribed (High-Capacity kit, Life Technologies, UK). RT-qPCR was performed using Taqman gene expression assays as follows: 0.5  $\mu\text{L}$  cDNA was amplified using 5  $\mu\text{L}$  TaqMan master-mix, 3.5  $\mu\text{L}$  nuclease-free water and 0.5  $\mu\text{L}$  TaqMan gene probes for Hp (Hs00605928\_g1, FAM). As reference control 0.5  $\mu\text{L}$  of  $\beta$ -2-microglobulin were used (Hs00984230\_m1, VIC) (Applied Biosystems). Reactions were performed using thermal cycles of 50°C (2 min) and 95°C (10 min); then 40 cycles of 15 s at 95°C, followed by 1 min at 60°C. The threshold cycle (Ct) was normalized against the reference gene and then for IL-6 stimulated cells. Fold changes in expression relative to unstimulated controls was calculated by using the formula  $2^{-\Delta\Delta\text{Ct}}$ . For mRNA expression of Hp by cancer cells, gene expression was normalized to the abundance of the reference gene transcript,  $\beta$ -2-microglobulin.

### Statistical analysis

Statistical analyses were performed with IBM SPSS Statistics 19. The Wilcoxon's test was employed to evaluate Hp expression in healthy vs. tumour tissue, or in cytoplasmic vs. membrane fractions. The Chi-squared test or Fisher's exact probability test were used to analyse the association between Hp expression in CRC tissue and standard clinicopathological features of patients and tumours. Results were considered statistically significant when  $p \leq 0.05$ .

## Results

### Immunohistochemical expression of Hp and association analysis with clinicopathological features of patients and tumours

The screening of Hp expression by IHC showed the

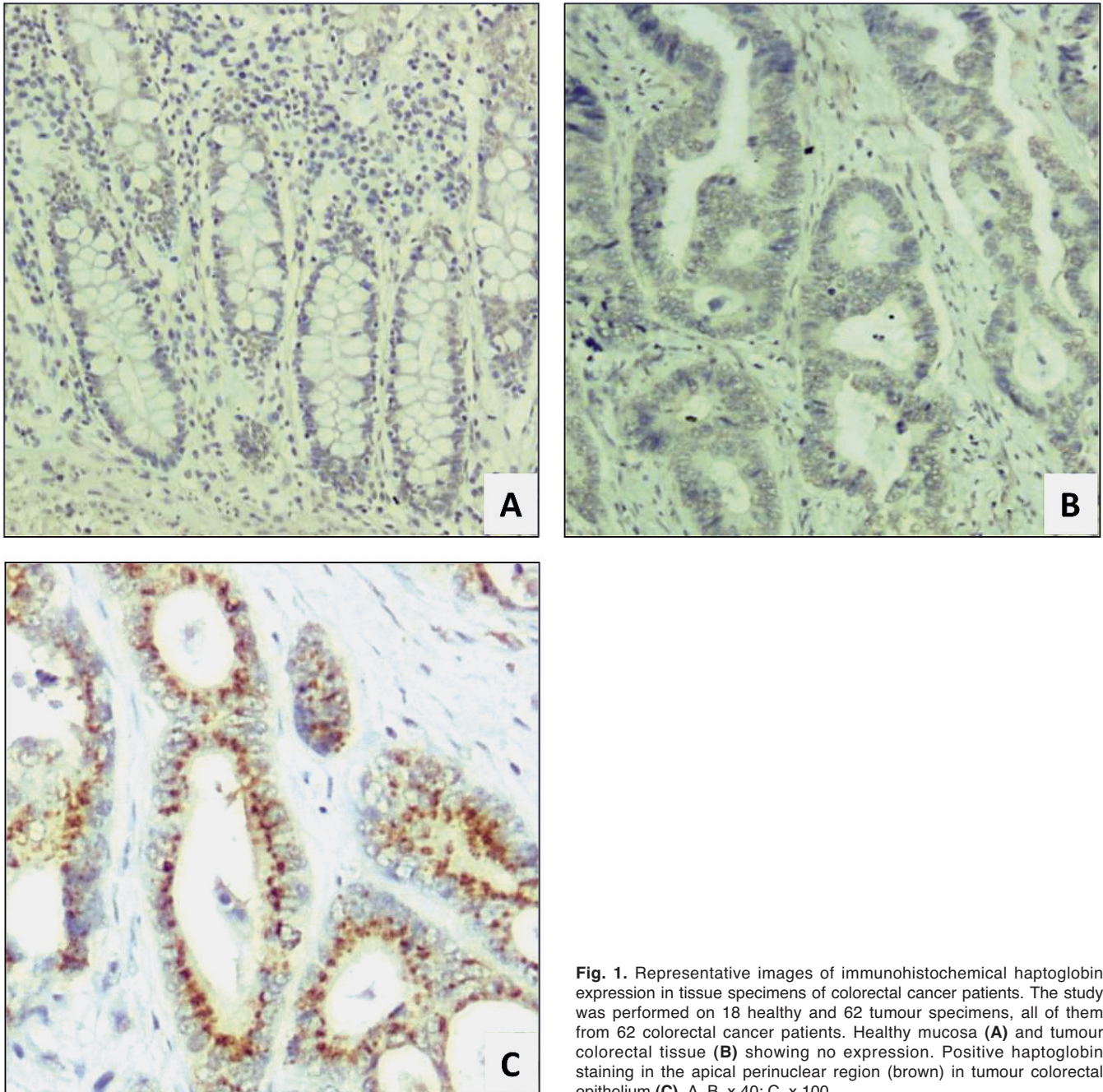


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presence of Hp in 15 of the 62 (24%) tumour samples examined (Fig. 1B), whereas it was undetectable in the 18 healthy specimens analysed (Fig. 1A). The sections of normal liver tissue included as positive controls always showed clear Hp expression, while negative controls displayed no expression (images not shown). The Hp staining in CRC was usually granular, weak, focal and mostly located in the perinuclear region of the epithelial cells, specifically on their apical side (Fig.

1C). The results of the semi-quantitative scoring indicated weak staining in 10 of the positive cases (67%) and moderate in the remaining 5 specimens (33%). According to Wilcoxon's test, the expression of Hp in CRC tissue was statistically greater than that observed for healthy tissue when comparing positive expression vs. no expression cases ( $p=0.014$ ), as well as the degree of staining among Hp-positive specimens ( $p=0.023$ ).

An association analysis was performed to elucidate



**Fig. 1.** Representative images of immunohistochemical haptoglobin expression in tissue specimens of colorectal cancer patients. The study was performed on 18 healthy and 62 tumour specimens, all of them from 62 colorectal cancer patients. Healthy mucosa (A) and tumour colorectal tissue (B) showing no expression. Positive haptoglobin staining in the apical perinuclear region (brown) in tumour colorectal epithelium (C). A, B, x 40; C, x 100.

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the relation between Hp expression in CRC tissue and the clinicopathological features of patients and their tumours. For this purpose, the IHC results were analysed by comparing the distribution of Hp-positive vs. Hp-negative specimens, and by taking into account the degree of staining among Hp-positive specimens. In the first case, results showed that Hp expression in CRC sections was significantly associated with Dukes' stage ( $p < 0.001$ ) and the presence of metastasis (pM,  $p < 0.001$ ) (Table 1). Specifically, Hp expression was more common in metastatic (8/11, 73% of the cases) than in non-metastatic CRC specimens (7/51, 14% of the cases). Duke's stage and metastasis also showed statistical significance when considering the intensity of Hp expression ( $p = 0.002$  and  $p < 0.001$ , respectively) (Table 1). By contrast, no

significant associations with Hp expression were found for the rest of the clinicopathological features, although we observed a higher proportion of Hp-positive cases among patients older than 78 (37%) compared to those that were 78 or younger (19%).

### Expression of Hp in subcellular fractions from colorectal surgical resections

Hp was detected in cytoplasmic and membrane fractions from five CRC patients as a single, blurry and wide WB band of approximately 40-45 kDa (Fig. 2A). As expected, Hp was present with a similar molecular mass in all serum samples used as positive controls (Fig. 2A). According to Wilcoxon's test, no differences in Hp

**Table 1.** Association analysis of haptoglobin expression in colorectal tumour tissue with standard clinicopathological features of colorectal cancer patients and tumours.

Clinicopathological feature	Subgroups	Hp expression			Degree of Hp expression			
		No	Yes	p	0	1	2	p
Sex	Male	26	8	0.563	26	7	1	0.184
	Female	21	7		21	3	4	
Age (years)	<66	16	2	0.188	16	1	1	0.204
	66-78	19	6		19	3	3	
	>78	12	7		12	6	1	
Age' (years)	≤78	35	8	0.112	35	4	4	0.086
	>78	12	7		12	6	1	
Tumour size (cm)	<4.4	20	9	0.228	20	4	5	0.052
	≥4.4	25	6		25	6	0	
Growth pattern	Non-polypoid	21	10	0.148	21	7	3	0.380
	Polypoid	24	5		24	3	2	
Histological type (differentiation)	Poor	4	2	0.779	4	2	0	0.233
	Moderate	26	9		26	4	5	
Tumour location	Well	16	4	0.293	16	4	0	0.345
	Colon	33	9		33	7	2	
Tumour location'	Rectum	13	6	0.516	13	3	3	0.517
	Proximal colon	16	3		16	3	0	
Lymphovascular infiltration	Distal colon	17	6	0.497	17	4	2	0.538
	Rectum	13	6		13	3	3	
Dukes' stage	No	31	6	<0.001	31	4	2	0.002
	Yes	7	2		7	2	0	
pTNM classification	A	6	3	<0.001	6	2	1	0.002
	B	14	0		14	0	0	
	C	24	4		24	3	1	
	D	3	8		3	5	3	
pT (primary tumour extent)	pT1	0	0	0.398	0	0	0	0.589
	pT2	10	3		10	2	1	
	pT3	27	11		27	8	3	
	pT4	10	1		10	0	1	
pT 1-2 vs. pT3-4	pT1-2	10	3	0.615	10	2	1	0.994
	pT3-4	37	12		37	8	4	
pN (lymph node metastasis)	pN0	21	4	0.448	21	3	1	0.772
	pN1	13	5		13	3	2	
	pN2	13	6		13	4	2	
pN0 vs. pN1-2	pN0	21	4	0.175	21	3	1	0.433
	pN1-2	26	11		26	7	4	
pM (distant metastasis)	pMX	44	7	<0.001	44	5	2	<0.001
	pM1	3	8		3	5	3	

Natural numbers represent number of cases. Decimal numbers represent p-values of Chi-squared or Fisher's exact probability tests. The age intervals correspond to the 33rd and 66th percentiles of the variable.

expression were noticed between healthy and tumour tissue. However, significantly more Hp expression was detected in cytoplasmic than in membrane fractions from both healthy ( $p=0.043$ ) and CRC tissue ( $p=0.043$ ) (Fig. 2B). Similarly, of the 5 CRC cases tested by WB, the 2 with the highest Hp expression showed positive tissue expression of the protein by IHC, while the remaining corresponded with 3 negative cases (data not shown).

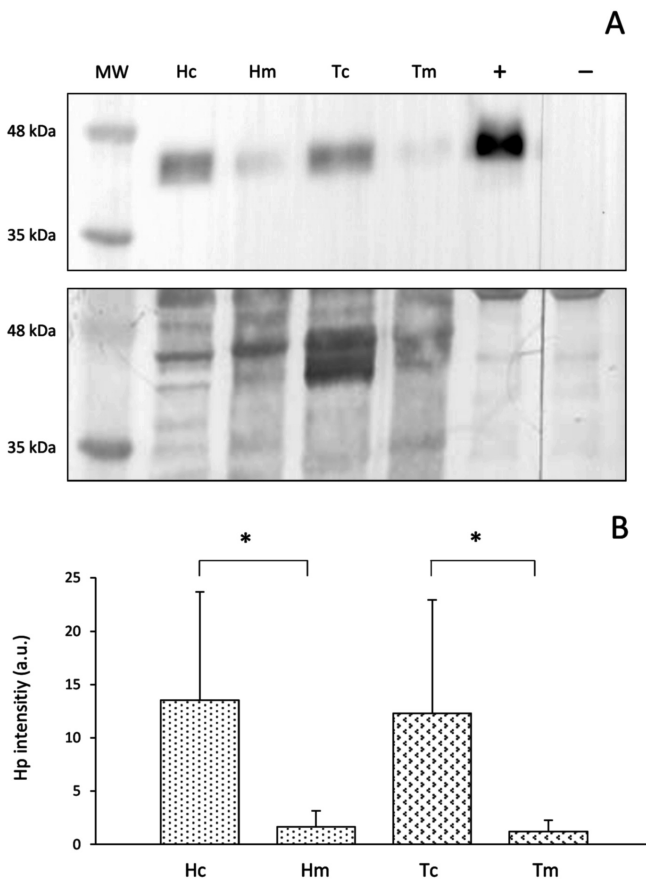
Expression of Hp in colorectal cancer cell lines

In order to verify the expression of Hp detected in the colorectal tissue of CRC patients, we carried out the immunodetection of the protein in cell extracts from Caco-2 and HT-29 CRC lines. The screening of Hp through WB was negative in both tumour lines (images

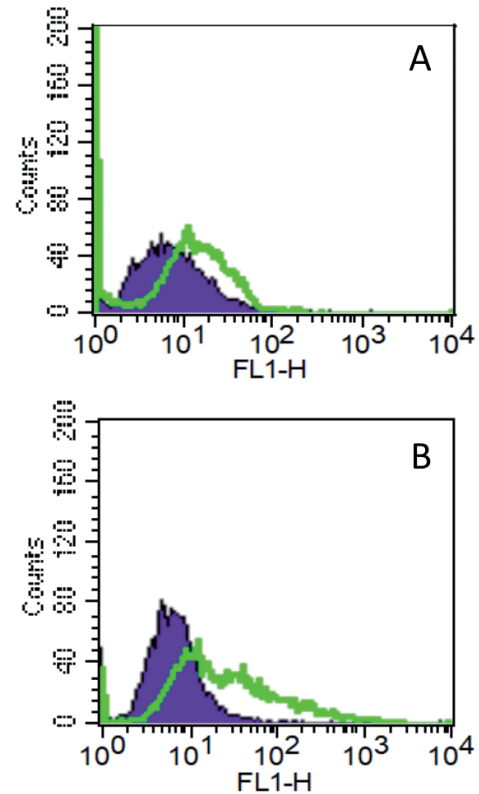
not shown). Next, we analysed the expression of the Hp protein by means of more sensitive fluorescence-based imaging techniques. The results showed that Caco-2 and HT-29 displayed significant intensity signals for Hp-stained cells when analysed by FC for cell surface expression, suggesting marked levels of Hp on their cell surface (Fig. 3). In the optimal conditions, i.e., using a non-enzymatic dissociation solution for cell detaching and gating the fluorescence of living cells exclusively, the signal was roughly twice as intense in HT-29 as in Caco-2 cells.

Additionally, IF microscopy analysis showed positive Hp expression in the same CRC cell lines, thus confirming the FC data. The fluorescence signal detected was weak and located on the cell surface of fixed and permeabilized cells (Fig. 4).

In view of the fact that, unlike WB, the imaging techniques showed clear signals of Hp expression, we decided to screen for Hp mRNA by quantitative PCR (qPCR). The results (Fig. 5A) corroborated the expression of *Hp* gene in liver HepG2 cells (Ct 18.3) and



**Fig. 2.** Expression of haptoglobin in colorectal cancer specimens by Western blotting. **A.** Representative example of Hp expression in crude cytosolic and total membrane fractions from healthy and colorectal tumour tissue (upper part) and Coomassie staining of the membrane (lower part). **B.** Quantification of Hp expression of five colorectal cancer patients. Legend: MW, molecular mass markers; Hc, cytoplasmic fraction from healthy tissue; Hm, membrane fraction from healthy tissue; Tc, cytoplasmic fraction from tumour tissue; Tm, membrane fraction from tumour tissue; +, serum positive controls; -, negative controls (without anti-Hp antibody); a.u., arbitrary units; Results are expressed as mean  $\pm$  SD. \*,  $p<0.05$  according to the Wilcoxon's test.



**Fig. 3.** Detection of cell surface expressed Hp by flow cytometry in colorectal cancer cell lines. **A.** Caco-2 cells. **B.** HT-29 cells. Cells were detached from tissue culture flasks by a non-enzymatic dissociation solution, stained with monoclonal antibody directed against Hp (green line histogram) or isotype control (blue filled histogram). Non-viable cells were removed from the flow cytometric analysis using TOPRO-3 for live/dead cell gating. Data herein showed are representative of the three independent experiments performed.

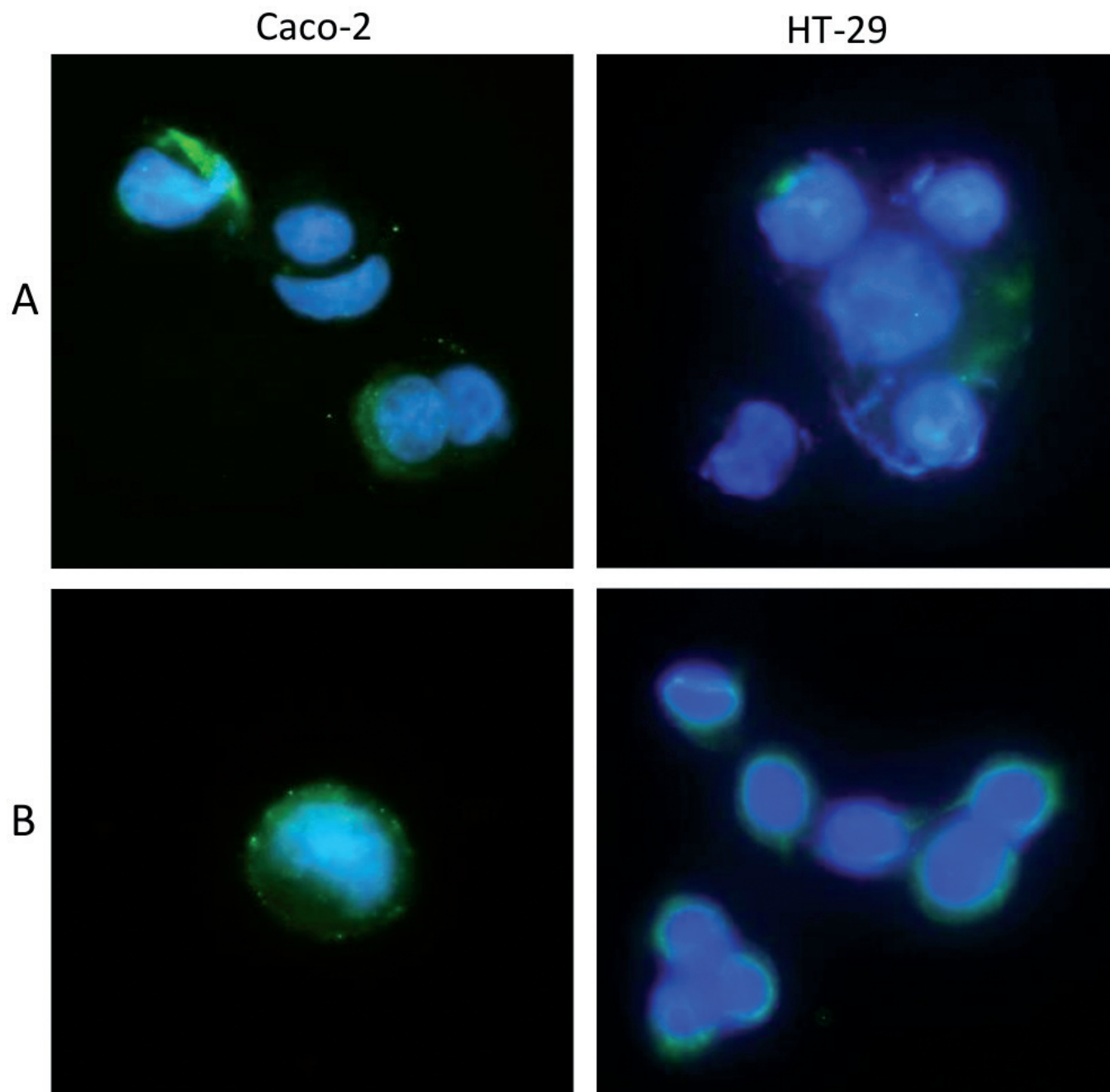


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both CRC cell lines, as well as a greater content of mRNA for Hp in Caco-2 (Ct 22.8) than in HT-29 (Ct 30.7) cells. Likewise, unlike the HepG2 liver cells used as positive control (Fig. 5B), the Hp expression in Caco-2 (Fig. 5C) and HT-29 (Fig. 5D) CRC lines was not inducible by the cytokine IL-6.

### Discussion

Hp is an acute-phase protein, mainly synthesised in hepatic cells and released into the plasma, where it acts as a haemoglobin scavenger (Wang et al., 2001). However, much evidence indicates that Hp is also



**Fig. 4.** Detection of Hp by immunofluorescence in the colorectal cancer cell lines Caco-2 and HT-29. Cells were cultured overnight on glass slides at a density of 25,000 cells/well and then fixed in 2% paraformaldehyde or permeabilized in freezing cold methanol. **A.** Cell surface expression on fixed cells. **B.** Negative intracellular expression, but positive cell surface expression in permeabilized cells. Hp signal is shown in green; nuclei are blue (DAPI-stained). Images shown are representative of the three independent experiments performed.

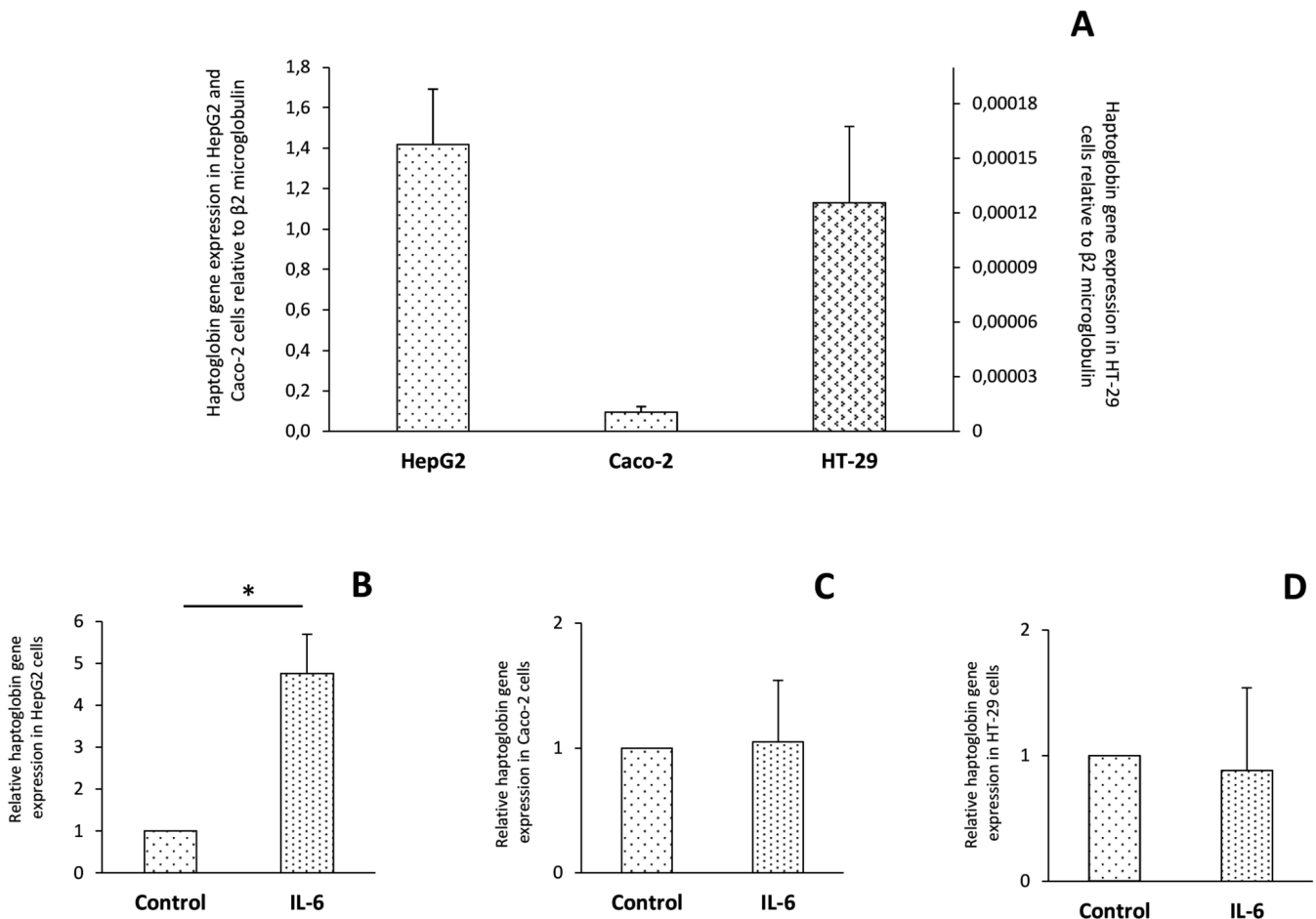
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expressed in other tissues such as lung, skin, spleen, brain, intestine, arterial vessels and kidney (Galicía and Ceuppens, 2011), which suggests that this protein may be involved in other biological processes (Edwards et al., 1986). Indeed, Hp has been shown to prevent oxidative stress, play a role in angiogenesis and form part of the innate immune response against pathogenic bacteria (Galicía and Ceuppens, 2011). Moreover, serum Hp polymorphism has been linked to an increased risk of inflammatory and autoimmune diseases, atherosclerosis and depression (Langlois and Delangue, 1996). Importantly, Hp has been suggested to confer protection to cancer cells from the activity of the immune system (Israel et al., 1981).

In accordance with previous evidence (Harvey et al., 2009), by IHC we detected Hp exclusively in the tumour cells from CRC patients. The staining was specifically located on the apical side of the perinuclear region of tumour epithelial cells (Fig. 1C), in the same way as

previously shown in lung adenocarcinoma and bronchi (Abdullah et al., 2009). Unlike other studies on CRC tissue (Harvey et al., 2009; Sun et al., 2012), the signal was weak and focal, possibly due to differences between our antibody and theirs.

The expression of Hp in the tumour tissue of CRC patients was statistically associated with tumour metastasis (Dukes' stage and pM from TNM classification) (Table 1). Therefore, our results from Caucasian population indicated that Hp may be related with the invasion and metastasis of CRC, in agreement with the only other comparable study (Sun et al., 2012), performed with Chinese population, that reported an increased expression of Hp in metastatic CRC specimens and its association with tumour infiltration and lymph node involvement (pT and pN from TNM staging, respectively). These authors proposed the use of Hp, together with osteopontin, as a predictor of hepatic metastasis. In line with this assumption, a recent study



**Fig. 5.** Detection of haptoglobin gene expression by qPCR. **A.** Haptoglobin mRNA amount in Caco-2 and HT-29 cancer colorectal cells relative to the reference gene,  $\beta$ -2-microglobulin. **B-D.** Fold change in gene expression of haptoglobin in HepG2, Caco-2 and HT-29 cells following stimulation with 100 ng/mL IL-6 for 3 h compared to unstimulated controls. Data are mean  $\pm$  SD of three independent experiments. \*,  $p < 0.05$  according to the Wilcoxon's test.



has shown that Hp promotes migration and invasion in LOVO and SW620 CRC cell lines, as well as the competence for tumour generation of subcutaneously-injected SW620 cells into athymic mice (Sun et al., 2016). Although little is known about the molecular mechanism underlying the role of Hp on migration and invasion of CRC, it has been reported that ovarian tumour cells exposed to Hp undergo similar morphologic rearrangements of the actin cytoskeleton as those of the epithelial-mesenchymal transition during malignant transformation (Garibay-Cerdenares et al., 2015).

Unlike IHC results, by WB we detected similar levels of Hp expression in membrane and cytosol fractions from healthy and CRC tissue (Fig. 2). The sample preparation for WB assays and the sensitivity of molecular assessments, probably made it possible to report by WB the undetectable Hp levels by histological examination of tissue sections. Accordingly, WB enabled the detection of Hp expressed in both epithelial and stromal cells, as well as serum Hp derived from the blood infiltration of the tissue by vascular permeability (Harvey et al., 2009). The difference in Hp abundance between the cytoplasmic and the membrane fractions is however noticeable (Fig. 2B).

*In vitro* analysis by fluorescence-based immunoassays showed expression of Hp in Caco-2 and HT-29 CRC cell lines, specifically at their cell surface. Moreover, qPCR demonstrated the expression of the *Hp* gene (Fig. 5A) and corroborated the positive results found with FC and IF. In this regard, it has been reported that vitamin D3 is able to induce (~4 fold) the expression of the *Hp* gene in Caco-2 cells (Claro da Silva et al., 2016). Even so, to the best of our knowledge, this is the first report finding direct evidence of Hp expression in Caco-2 and HT-29 cells, which has been previously found in other CRC cell lines such as SW480, SW620 and COLO205 (Park et al., 2010). Additionally, the expression of the *Hp* gene in the two CRC lines was not inducible by the pleiotropic cytokine IL-6, unlike HepG2 liver cells (Westfall et al., 2013) used as positive control in the qPCR assay (Fig. 5B-D). In this regard, it has been recently reported that colonic (myo)fibroblasts are the main source of IL-6 in the colorectal tumour tissue and are responsible for stemness maintenance and the inflammatory response that stimulate CRC progression (Huynh et al., 2016), which points out the importance of tissue microenvironment in the persistence of pro-survival stimuli. In the same sense, it has been reported that IL-6 does not alter its expression after the malignization of the colorectal tissue and that IL-6 synthesis by Caco-2 cells requires stimulation with the proinflammatory cytokine TNF $\alpha$  (Malicki et al., 2009), also that IL-6-treated Caco-2 cells modulated the mRNA of several genes involved in tumour invasion and apoptosis, while the *Hp* gene expression was unaffected (Pucci et al., 2009).

In a recent study, we identified Hp as a putative CDw75-bearing glycoprotein in colorectal tissue specimens from CRC patients (Mariño-Crespo et al.,

2018). Although those results did not confirm the epithelial origin of the CDw75-positive Hp, it has been reported that at least serum Hp possesses this epitope (Park et al., 2010). Here we provide some evidence that Hp is expressed by the tumour colorectal epithelium, in agreement with previously published reports (Uhlén et al., 2005; Harvey et al., 2009; Sun et al., 2012). Taking into account the ability of Hp (Hanasaki et al., 1995) and CDw75 (Nitschke, 2005) to interact with the inhibitory B cell receptor CD22, and the fact that this receptor specifically recognises  $\alpha(2,6)$ linked sialic acids (Stamenkovic et al., 1991; Sgroi et al., 1993), we hypothesize that CDw75 could account (at least in part) for the suggested immunosuppressive action of Hp (Israel et al., 1981). The presence of Hp in the CRC epithelium, but not in the healthy tissue, strengthens the assumption that the ectopic expression of this protein by CRC cells may represent a mechanism of immune surveillance evasion through the CDw75-CD22 interaction and the subsequent inactivation of B cells. Nevertheless, future studies on this matter are required in order to test this hypothesis.

The question of whether the overexpression of Hp in colorectal tumour tissue is linked to a possible Hp secretion from CRC to the bloodstream arises from this work. In fact, a compatible mechanism is known to happen in neutrophils and monocytes, where Hp can be found as intracellular granules (Wagner et al., 1996) that are secreted in response to TNF $\alpha$  stimulation (Berkova et al., 1999). This pattern of expression resembles the perinuclear staining we observed by IHC in tumour cells. Given that the usual location of the Golgi apparatus is also perinuclear, our observations may indicate the existence of a Golgi-mediated secretion pathway of Hp in CRC epithelial cells. However, to elucidate the existence of this putative secretory pathway additional studies must be carried out.

In conclusion, this study reports *in vivo* and *in vitro* evidence of cell membrane and intracellular Hp expression by CRC cells. Additionally, it provides support to the association between Hp expression and a higher tumour invasion and propagation potential of CRC. The consideration of all these findings suggests that Hp represents a good target for functional studies aimed at clarifying the role it plays in CRC carcinogenesis and progression, as well as its possible usefulness in the design of new therapeutic strategies for this disease.

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