Different iron-handling in inflamed small and large cholangiocytes and in small and large-duct type intrahepatic cholangiocarcinoma

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Cholangiocarcinoma (CCA) represents the second most common primary hepatic malignancy and originates from the neoplastic transformation of the biliary cells. The intrahepatic subtype includes two morpho-molecular forms: large-duct type intrahepatic CCA (iCCA) and small-duct type iCCA. Iron is fundamental for the cellular processes, contributing in tumor development and progression. The aim of this study was to evaluate iron uptake, storage, and efflux proteins in both lipopolysaccharide-inflamed small and large cholangiocytes as well as in different iCCA subtypes. Our results show that, despite an increase in interleukin-6 production by both small and large cholangiocytes, ferroportin (Fpn) was decreased only in small cholangiocytes, whereas transferrin receptor-1 (TfR1) and ferritin (Ftn) did not show any change. Differently from in vitro models, Fpn expression was increased in malignant cholangiocytes of small-duct type iCCA in comparison to large-duct type iCCA and peritumoral tissues. TfR1, Ftn and hepcidin were enhanced, even if at different extent, in both malignant cholangiocytes in comparison to the surrounding samples. Lactoferrin was higher in large-duct type iCCA in respect to small-duct type iCCA and peritumoral tissues. These findings show a different iron handling by inflamed small and large cholangiocytes, and small and large-duct type iCCA. The difference in iron homeostasis by the iCCA subtypes may have implications for the tumor management.

Key words: Inflammation; cholangiocytes; cholangiocarcinoma; iron-proteins; ferroportin; hepcidin; lactoferrin.

ABSTRACT

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Informed consent: The manuscript does not contain any individual person’s data in any form.
Introduction

Cholangiocytes are simple epithelial cells that line the intrahepatic biliary tree, a complex three-dimensional network of tubular canals of different diameters within the liver. Small and large cholangiocytes reside primarily in small and large intrahepatic bile ducts, respectively, with different functions and responses to injury. In the human biliary tree, cholangiocytes are damaged in a variety of diseases, namely cholangiopathies, which often cause advanced liver failure. It is well-known the role of cholangiocytes in liver regeneration when hepatocyte regrowth is compromised, whereas it is still under study the role of biliary cells in innate and adaptive immune responses.

In recent years, the incidence of hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) has increased. Particularly, CCA is a devastating adenocarcinoma arising from malignant transformation of cholangiocytes and represents a heterogeneous group of malignant neoplasms. They are anatomically classified as intrahepatic (iCCA) (arising proximal to the second order bile ducts), perihilar (pCCA) (arising between the second order bile ducts and the insertion of the cystic duct into the common bile duct) and distal extrahepatic (dCCA) (arising between the insertion of the cystic duct and the ampulla of Vater). In addition to this anatomical classification, other factors such as tumor growth pattern and the cell of origin provide alternative methods of classification that may better predict tumor invasion and progression. ICCA is characterized by two histological subtypes: originating either from small or large intrahepatic bile ducts. The first type is called small-duct type iCCA showing an almost exclusively mass-forming growth pattern, whereas the second type is a mucinous iCCA, called large-duct type and appears as mass-forming, periductal infiltrating or intraductal growing. This histological subdivision of iCCA is of great importance because the different cell of origin reflects the diversities in risk factors, molecular profile, clinical outcome and response to treatments.

Several efforts have been made to decipher the molecular mechanisms and the concurrent factors involved in cancer development and proliferation and, among these, iron represents an important risk factor.

Iron is an essential element for living cells as component of fundamental biological processes such as DNA replication and energy production. However, iron can also be toxic when present in excess because of its capacity to generate reactive oxygen species (ROS), which can result in macromolecule damage, tissue injury and organ failure. As iron is extremely insoluble, mammals have evolved different iron-binding proteins, such as lactoferrin (Lf), ferritin (Ftn) and transferrin (Tf), to favor, respectively, its absorption, storage and delivery. Moreover, different iron transport systems ensure iron to be sorted among tissues, blood and secretions in a safe manner. In particular, the interface between systemic and cellular iron homeostasis mainly involves the cellular iron acquisition by Tf-bound iron through Tf receptor 1 (TfR1)-mediated endocytosis, whereas, only for particular iron-recycling cells, such as enterocytes, hepatocytes and macrophages, intracellular iron can be (re)-exported into the plasma by the iron export complex, involving a permease, namely ferroportin (Fpn), and a ferroxidase. Indeed, systemic iron homeostasis relies on the concerted action of peculiar cell types, i.e., enterocytes, macrophages and hepatocytes, able to handle iron in the way to quickly respond to physiological demand or to pathological stimuli and conditions, including inflammation and cancer. In inflammatory conditions, usually associated to cancer development and progression, intracellular iron load increases due to the inactivation of the iron efflux system, mainly related to the interleukin (IL)-6-mediated overexpression of hepcidin, a liver-secreted hormone responsible for Fpn degradation. This scenario, intracellular iron overload assumes a synergic role in promoting carcinogenesis, enabling cancer cells to exploit aerobic metabolism for proliferation, migration, invasion of surrounding tissues and metastatization. Therefore, it is not surprising that expression profiles of most of the iron-handling proteins result altered in many different types of tumors.

Even if the liver is the central organ for iron homeostasis, little is known about iron and cholangiocytes in healthy and pathological states, especially regarding the two subpopulations of small and large cholangiocytes. Therefore, our aim was to understand whether the anomalous expression of the major proteins involved in iron homeostasis could play a role in CCA development and progression. The in vitro experiments were conducted in murine small and large cholangiocytes stimulated with LPS, of which the expression of Fpn, cytosolic Ftn, TfR1, and IL-6 have been evaluated. The in vivo study was conducted in iCCA and peritumoral noncancerous tissue, of which iron uptake (TfR-1 and Lf), iron storage (Ftn), and iron efflux (Fpn and hepcidin) proteins were analyzed.

Materials and Methods

Materials

Reagents were purchased from Abcam (Cambridge UK), Sigma (St. Louis, MO, USA) and DBA Italia srl (Milan, Italy) unless otherwise indicated. The mouse monoclonal Lf antibody was obtained from Abcam (ab10110), the rabbit polyclonal Ftn heavy chain (H-Ftn) antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA; sc-25617), the mouse monoclonal TfR1 antibody from Santa Cruz Biotechnology (sc-65882), the mouse monoclonal Fpn antibody was generously provided by T. Arvedson (Agenon), and the mouse monoclonal H-Ftn antibody from Santa Cruz Biotechnology (sc-100277). The LSAB System-HRP used for IHC was purchased from Dako-Agilent (Santa Clara, CA, USA), while to develop the immunoreaction DAB + substrate chromogen system (Dako Agilent) was used. ELISA kit for IL-6 quantification was obtained from BioLegend, USA. In the end, Schiff’s reagent for histology was purchased from Carlo Erba (Milan, Italy; #477591).

Cell lines

In vitro studies were performed in immortalized small and large cholangiocyte lines, which display morphological and functional characteristic similar to that of freshly isolated small and large cholangiocytes (gifts from Prof. G. Alpini, Indiana University, School of Medicine, Indianapolis, IN, USA). The cell lines were maintained in minimum essential medium (Invitrogen, Carlsbad, CA, USA) containing 10% heat-inactivated FBS, 1% penicillin and streptomycin, and 2 mmol/L l-glutamine at 37°C in a humidified 5% CO2 incubator. We have previously shown that these cells display morphological (difference in size and transepithelial resistance), phenotypic (expression of cytotokeratin-19), and functional features (cAMP response in large but not small cholangiocytes), similar to freshly isolated small and large mouse and rat cholangiocytes.

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Stimulation of cholangiocytes with LPS

Small and large cholangiocytes were seeded in 25 cm² flasks at a density of approximately 10⁵ cells/mL in 5 mL of the specific medium for 48 h, at 37°C in an atmosphere of 95% air and 5% CO₂. After 48h, cells were washed twice with 5 mL of PBS without calcium and magnesium and treated with 1 or 100 µg/mL LPS from E. coli (InvirosGen, San Diego, CA, USA). The stimulations were carried out for 48 h in an atmosphere of 95% air and 5% CO₂ at 37°C. After 48 h of incubation, the supernatants were harvested, aliquoted, and stored at −80°C for cytokine quantitation. The cells were scapped in 2 mL of PBS containing 1 mM phenylmethylsulfonyl fluoride (PMSF), pelleted by centrifugation at 2,500 × g for 5 min, and stored at −80°C for protein analysis.

Cytokine analysis

Quantitation of IL-6 was performed on cell monolayer supernatants of small and large cholangiocytes by ELISA, using murine ELISA Max Deluxe Sets (BioLegend, San Diego, CA, USA).

Western blots

Small and large cholangiocytes (about 5 × 10⁶ cells) were lysed in 300 µL of lysis buffer (25 mM 3-morpholinopropanesulfonic acid pH 7.4/150 mM NaCl/1% Triton containing 1 mM PMSF, 2 µM leupeptin, and 2 µM pepstatin) in ice for 1 h. Total protein content of samples was measured by Bradford assay. For SDS-PAGE, 20 µg of total protein in SDS sample buffer containing 1,4-dithiothreitol, were heat-treated (except for Fpn) and loaded. For Western Blot analysis, primary antibodies were diluted in PBST at following concentration: anti-Fpn (1:10,000), anti-TIR1 (1:5,000), anti-H-Ftn (1:10,000) and anti-actin (1:10,000). After incubation with the appropriate secondary Horseradish Peroxidase-conjugated antibody, blots were developed with Enhanced Chemiluminescence (ECL Prime; GE Healthcare, Amersham, UK).

Protein bands were quantified by densitometry analysis, with Image J and proteins levels were normalized by β-actin.

Human samples

Liver samples of fifteen human iCCA and peritumoral non-cancerous tissue were obtained from patients submitted to surgical resection (aged 50 to 83 years) with iCCA presenting as a single mass lesion within the liver, after informed consent, at the Department of Gastroenterology of the Academic General Hospital Umberto I, Sapienza University of Rome in accordance with the ethical standards and the Helsinki Declaration of 1975.

In particular, iCCA can arise at any point of the intrahepatic biliary tree, from the second-order bile ducts. Furthermore, iCCA can be divided into two main subtypes according to the histological level. Small bile duct iCCA originates from small intrahepatic bile ducts and with minimal mucin production. Large bile duct iCCA arises in large intrahepatic bile ducts and contains mucin-producing columnar tumor cells.⁷⁷

Light microscopy: morphologic study

Human samples were immediately fixed in 10% buffered formalin and then dehydrated with alcohol, cleared in xylene and embedded in paraffin wax. Afterwards, the tissues were serially sectioned, obtaining 3-µm-thick sections that were stained with Periodic Acid-Schiff staining system (PAS). This method is used for detection of glycogen and mucin in tissues, which were stained purple, while the nuclei were stained blue. Sections were deparaffinized and rehydrated. Following washing with PBS, they were incubated with 0.5% periodic acid solution for 5 min, then stained with Schiff’s reagent for 15 min, followed by counterstaining with hematoxylin solution for 2 min. All steps were performed at room temperature, and cells were rinsed with tap water after each step. The final morphological analysis has been performed using a light microscopy Leica Microsystems DM 4500.

Light microscopy: immunohistochemistry

As previously described, human specimens were immediately fixed, dehydrated with alcohol, cleared in xylene, embedded in paraﬁn wax, and sectioned. For immunohistochemistry (IHC), endogenous peroxidase activity was blocked by incubation in hydrogen peroxide (3%). Sections were then incubated overnight at 4°C with the different antibodies (Lf, Ftn, TIR1, Fpn and hepcidin). Samples were rinsed with 1x phosphate buffered saline (PBS), incubated with secondary biotinylated antibody and then with Streptavidin-HRP (LSAB+ System-HRP). Diaminobenzidine (DAB) was used as chromogen, and sections were counterstained with Hematoxylin. For all immunoreactions, negative controls were also included. Immunohistochemical observations were taken by a light microscopy Leica Microsystems DM 4500, with a camera JenoptikProgRes C10 Plus (Jena, Germany). In addition, all slides were scanned by a digital scanner (Aperio Scanscope CS System, Aperio Digital Pathology; Leica Biosystems, Milan, Italy) and processed by ImageScope software. We calculated the area occupied by Lf, Ftn, TIR, Fpn and hepcidin-positive cholangiocytes in the several iCCA and peritumoral samples. The quantifications were carried out by an image analysis algorithm and expressed as the percentage of the total area.⁵⁷

Statistical analysis

All experiments were run at least in triplicate. Results are expressed as mean ± SE. Statistical analysis was performed with GraphPadPrism and analysis of variance was used to compare quantitative data populations with normal distribution and equal variance.

Results

Iron proteins’ changes in small and large cholangiocytes upon LPS treatment

To induce a putative inflammatory response in both small and large cholangiocytes, the cultures were stimulated with 1 or 100 µg/mL LPS. As shown in Figure 1A, the levels of IL-6 significantly increased upon treatment with both 1 and 100 µg/mL of LPS compared to un-stimulated small or large cholangiocytes. In particular, the IL-6 synthesized by small cells stimulated with 1 or 100 µg/mL LPS increased by 2.5 folds with respect to unstimulated ones. On the other hand, large cholangiocytes were more responsive to high-LPS concentration, resulting in an increase by 3.5 folds in IL-6 production. Concerning iron-handling proteins, only small cholangiocytes showed a significant down-regulation in Fpn expression following 100 µg/mL LPS challenge (Figure 1B). Regarding the large sub-population, even no significant difference in Fpn levels was recorded for both inflammatory challenges, a decreasing trend was observed upon high dose LPS (Figure 1B). Concerning Ftn and TIR1, both small and large cholangiocytes did not show any significant difference in their expression patterns upon stimulation with both high and low LPS concentrations (Figure 1C,D).

Tissue morphology and expression analysis of iron-related proteins in human iCCA

The morphological aspects of the different hepatic samples were evaluated by PAS (Figure 2 A-D). Through this staining we differentiated six large-duct type iCCA, PAS positive and nine samples as small-duct type iCCA, PAS negative. The architecture
of the peritumoral tissues, both from large-duct and small-duct type iCCA, shows the typical hepatic aspect, with cords of hepatocytes, presence of portal spaces and enlargements of the sinusoidal network (Figure 2 A, C). The diffused purple color represents the physiologic amount of glycogen deposition in hepatocytes. The large-duct type iCCA sample changes totally with absence of the normal hepatic morphology, cholangiocytes form large gland-like structures, that are composed of cuboidal to cylindrical cells characterized by a most pronounced mucin production (largest biliary ducts PAS positivity) as evident with the widespread purple color (Figure 2B). The small-duct type iCCA subtype was characterized by areas of small tubular cord-like structures and irregular lumina, mainly formed by mucin-negative cholangiocytes (smallest biliary ducts PAS negative) (Figure 2D).

To assess whether iron proteins are altered in iCCA, we analyzed the expression of Fpn, hepcidin, TIR1, and Ftn through immunohistochemistry. We have evaluated and quantified the protein expressions in peritumoral and tumoral tissues. Fpn expression was found increased in large-duct type iCCA when compared to the surrounding tissues, whereas no significant difference was recorded in small-duct type iCCA (Figure 3A). Although Fpn increased in large-duct type, surprisingly, hepcidin expression was raised in both iCCAs compared to the peritumoral tissue (Figure 3B). Furthermore, the expressions of Ftn and TIR1 were significantly increased in small-duct type iCCA, the tumor arising from non-mucin-secreting cholangiocytes lining small immature bile ducts, compared to cholangiocytes in surrounding liver, while we found a moderate enhancement in large-duct type iCCA, the tumor deriving from the mucin-secreting epithelium lining the large differentiated ducts (Figure 3 C, D). Lastly, Lf immunopositivity has been found up-regulated in large-duct type iCCA compared to small-duct type iCCA and peritumoral tissue (Figure 3E). The quantitative values of Fpn, hepcidin, TIR1, Ftn and Lf are reported in Table 1.

Discussion

Liver is one of the primary organs involved in the maintenance of iron homeostasis. In this respect, iron-handling by hepatocytes in physiological, inflamed and tumor states, has been extensively studied in the last decade, only few studies have investigated the dysregulation of iron metabolism in CCA models in vitro as well as in CCA patients. However, no data on the differential role of small and large cholangiocytes in iron-handling, in either physiological or pathological conditions, has been reported. Here, we show, in an in vitro model of immortalized small and large cholangiocytes challenged with LPS, that both sub-populations remain very poorly responsive to the inflammatory stimulation regarding the iron-related proteins. In particular, though significant up-regulation of IL-6 was recorded for both small and large cells, no significant variation in the expression of Ftn and TIR1 was observed. Conversely, Fpn showed a significant down-regulation only in small cholangiocytes upon high dose LPS (Figure 1B). Even if large cholangiocytes did not modulate Fpn synthesis under inflammatory stimuli (Figure 1B), the results found with small cholangiocytes are in agreement with most of the studies on iron proteins in inflamed models, all showing Fpn to be regulated upon inflammatory challenges. In particular, Fpn is down-regulated in

Table 1. Measurement of the percentage of cholangiocytes positive for the expression of ferroportin (Fpn), hepcidin, transferrin receptor 1 (TIR1), ferritin (Ftn) and lactoferrin (Lf), in the different liver sections.

<table>
<thead>
<tr>
<th></th>
<th>Surrounding liver</th>
<th>Small-duct type iCCA</th>
<th>Surrounding liver</th>
<th>Large-duct type iCCA</th>
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<tbody>
<tr>
<td>Fpn+ cholangiocytes</td>
<td>19.1 ± 1.6</td>
<td>28.6 ± 3.3*</td>
<td>19.3 ± 1.9</td>
<td>19.3 ± 2.9</td>
</tr>
<tr>
<td>Hepcidin+ cholangiocytes</td>
<td>16.1 ± 2.0</td>
<td>29.2 ± 2.0*</td>
<td>16.7 ± 2.2</td>
<td>30.9 ± 2.5*</td>
</tr>
<tr>
<td>TIR1+ cholangiocytes</td>
<td>16.5 ± 2.0</td>
<td>41.2 ± 1.0*</td>
<td>16.1 ± 2.7</td>
<td>24.8 ± 2.0*</td>
</tr>
<tr>
<td>Ftn+ cholangiocytes</td>
<td>18.4 ± 1.3</td>
<td>33.8 ± 2.5*</td>
<td>18.2 ± 1.9</td>
<td>26.3 ± 2.2*</td>
</tr>
<tr>
<td>Lf+ cholangiocytes</td>
<td>19.6 ± 0.7</td>
<td>17.8 ± 1.7</td>
<td>19.5 ± 1.6</td>
<td>29.1 ± 2.7*</td>
</tr>
</tbody>
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*P<0.001 (unpaired t-test)
inflamed macrophages of *in vitro* models, whereas it is up-regulated in WT and cystic fibrosis (CF) bronchial epithelium both *in vitro* and murine model of *Pseudomonas aeruginosa* infection. Therefore, it is emerging that Fpn can differently respond to infection or inflammation relating from its physiological role and from the *in vitro* or *in vivo* models. Indeed, Fpn acts as the only iron exporter from cell to blood in both hepatocytes and macrophages, by allowing both body iron to be stored/redistributed and hemoglobin-deriving iron to be recycled, respectively. Following infection or inflammatory stimuli, IL-6 up-regulation, both through hepcidin-dependent or independent pathways, leads to Fpn down-regulation, thus activating an unsafe intracellular iron overload which increases host susceptibility to infection or cancer proliferation. It is important to underline that the Fpn localization is also pivotal. As a matter of fact, in bronchial epithelium, Fpn, located on the cell apical side, acts as an iron detoxifying agent, able to translocate the metal in the bronchial lumen, thus allowing its further removal by the ciliary pathway. In this case, following infection/inflammatory stimuli Fpn is up-regulated, thus enhancing the activity of the detoxifying pathway thus facilitating the iron removal from bronchial lumen.

Here, in our *in vitro* model, only small cholangiocytes seem to respond to inflammatory challenge, down-regulating Fpn similarly to that observed in inflamed macrophages and differently to that found in WT and CF bronchial epithelia where Fpn is localized on apical side. Even more analysis should be carried out to confirm the response of the two cholangiocyte sub-populations to inflammatory stimuli, our data on Fpn expression may suggest a potential different role between small and large cholangiocytes in iron-handling during infection/inflammation. Indeed, small cholangiocytes, probably due to their immature nature, may respond in a more efficient way in this iron-management when damaged, in comparison to the mature and well-differentiated large cholangiocytes.

In this study, we have also investigated the important role of iron acquisition, sequestration and export in contributing to the development and progression of biliary cancer. Several and recent studies have underlined that the iron homeostasis is dysregulated in cancer. In fact, iron is essential for normal cell function and many tumor cells require an increased, acquisition of iron to sustain growth and proliferation. Here, according to other studies, in cancer cells from both small-duct and large-duct type iCCA, we have observed an increased expression of TIR1 to

Figure 2. Representative images of Periodic-Acid Schiff (PAS) staining of peritumoral tissue and CCA. A) The image shows a peritumoral area from large-duct type iCCA; it presents a typical morphology with a portal space and the cords of hepatocytes greatly stained in dark pink/purple for the high percentage of carbohydrate content such as glycogen and mucin (black asterisks). B) Characteristic image of large-duct type iCCA stained with periodic-acid Schiff, in which the normal hepatic architecture is destroyed; these tumors are formed by acinar or tubular structures and tumor cells are columnar with abundant cytoplasm; as the name suggests, this subtype of tumor is mainly composed of mucin-producing cells, as shown by the diffused typical PAS color (black asterisks). C) An additional image of peritumoral tissue, in this case from small-duct type iCCA; there are not present relevant differences among the several peritumoral areas, where the parenchyma could be characterized by enlargements of the sinusoids (red arrows). D) Periodic-acid Schiff stain in the other subtype of CCA: small-duct type iCCA; these tumors are formed by areas with small tubular cord-like structures with irregular cavities; the section shows histological differences compared to the mucin-subtypes mainly regarding the very few focal mucin producing regions. Scale bars: 100 µm.

Figure 3. Representative images of the immunohistochemical study in both iCCA subtypes and peritumoral tissues. A) Immunohistochemistry for ferroportin (Fpn) (red arrows), in this case, small-duct type iCCA has showed a higher immunopositivity compared the peritumoral tissue and large-duct type iCCA. B) Immunohistochemistry for hepcidin (red arrows), tumoral biliary cells have displayed an important increase of the protein compared the peritumoral hepatic tissue. C) Immunohistochemistry for transferrin receptor protein (TIR1) (red arrows), cholangiocytes from small-duct type iCCA were intensively marked by the immunostaining compared the surrounding samples, while there was a lower increase in large-duct type iCCA always in respect to the peritumoral tissues. D) Immunohistochemistry for ferritin (Ftn) (red arrows), there was enhanced expression of the protein in tumoral cholangiocytes compared with the surrounding cholangiocytes. E) Immunohistochemistry for lactoferrin (Lf) (red arrows), showing an enhanced expression in large-duct type iCCA compared to peritumoral tissues (for quantitative analysis, see Table 1). Scale bars: 50 µm.
enhance the intracellular iron availability. In relation to the increase of TIR1, we would have expected altered levels of Fpn, while we found similar expressions among peritumoral tissues and large-duct type iCCA (19.3±1.9 and 19.3±2.9), and a slight increase in small-duct type iCCA (28.6±3.3), probably due to the poorly defined nature of the cells. In fact, small-duct type originates from small cholangiocytes, a population of immature cells, that in a specific position, such as the Herin’s channels constitute the niche of hepatic progenitor cells (HPCs) able to differentiate into cholangiocytes and hepatocytes. Part of these cells could follow the hepatocyte lineage with a regular use of iron. Many tumor types exhibit an altered regulation of Fpn,48 its overexpression could be linked to the inhibition of metastasis, as reported in lung and liver, through reduction of typical epithelial-to-mesenchymal transition markers such as Snail1, Twist1 and vimentin.49 Thus, the different Fpn expression in the two iCCA subtypes could be linked to the progression of the damage, the small-duct type iCCA arising from an immature population of cells could induce different tumor outcomes. Moreover, Fpn overexpression in small-duct type iCCA, along with increased levels in TIR1 expression, could be related to increased iron-flux from the deeper ducts to the more superficial population of mucin-producing cells, which present TIR1 overexpression whereas basal level of Fpn. This may represent an intriguing mechanism through which small-cells could act as an iron gate to furnish extra-demand of the metal to a more aggressive large-duct type iCCA. Regarding Ftn, in some tumors, it is present in low concentrations, but its upregulation has been described in several other tumoral tissues.50 In our samples, we found an enhancement of the Ftn expression in the two subtypes of iCCA compared with the peritumoral hepatic tissue, which is probably linked to its increase in stromal and inflammatory cells, hence suggesting its putative implication in counteracting oxidative stress in iron-overloaded cancer cells.50,51 Conversely, it has been reported how hepcidin levels increase in myeloma, renal cell carcinoma, prostate and other cancers for its central role in systemic iron uptake and recycling.52-54 Large-duct type iCCA (30.9±2.5) and small-duct type iCCA (29.2±0.8) exhibit higher levels of hepcidin expression compared with the surrounding healthy liver in CCA patients (16.7±2.2). Furthermore, hepcidin expression has been positively linked with tumor stage55 and it is activated by IL-6, the latter found up-regulated in CCA tissues.56 Increased IL-6 levels, typical in a tumoral situation, are also associated with an enhanced Lf, which is known to display a plethora of activities, including the inhibition of carcinogenesis in different tumors, like brain, breast, esophagus, lungs and liver,57-59 as well as the anti-inflammatory activity against IL-6 expression in vitro and in vivo.60-65 We have previously showed that Lf and its receptors are present in biliary epithelium;66 we found an upregulation in experimental mouse models and in primary biliary cholangiocytes (PBC), suggesting an increased Lf uptake by damaged cholangiocytes.67 Our results show that Lf expression is higher in large-duct type iCCA compared with the small-duct type iCCA and the peritumoral tissues, suggesting high infiltration rates of neutrophils in the tumoral surrounding tissue and a possible supporting role of the Lf against the tumoral growth of large-duct type iCCA. The altered iron homeostasis shown here in small- and large-derived CCAs may be correlated to a global increase in iron flux and availability, underlined by elevated levels of Ftn and TIR1 in both subtypes of CCA as well as to normal or slight increased levels of Fpn in large-duct and small-duct type CCA, respectively.

In summary, although new experiments should be carried out and new markers evaluated, our data present novel peculiar aspects of iron-handling between small and large cholangiocytes and their associated iCCAs, showing different response to inflammatory stimuli and tumor-associated microenvironment. Indeed, iron-related proteins in immortalized cells seem to poorly respond to IL-6 signal-

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