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ORIGINAL ARTICLE

Immunohistochemical Assessment of the Expression of Biliary Transportation Proteins MRP2 and MRP3 in Hepatocellular Carcinoma and in Cholangiocarcinoma

Cinthya Santos Cirqueira[1,2] · Aloisio Sousa Felipe-Silva[2] · Alda Wakamatsu[2] · Lidiane Vieira Marins[2] · Eziel Cavalcanti Rocha[2] · Evandro Sobroza de Mello[2] · Venâncio Avancini Ferreira Alves[2]

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Abstract

Multidrug resistance-associated protein 2 (MRP2) is a multi-specific organic anion transporter predominantly expressed in the canalicular membrane of hepatocytes, epithelial cells from gallbladder and apical membranes of proximal tubular kidney epithelium whereas multidrug resistance-associated protein 3 (MRP3) is present in the basolateral membrane of hepatocytes and cholangiocytes. This study aims to detect the expression of these transporters in hepatocellular carcinoma (HCC) and in cholangiocarcinoma (CC), searching for evidences for future studies on differential diagnosis and on clinical essays. The immunohistochemical reactivity (IHC) of these transporters was assessed in tissue microarrays of 80 HCC and 56 CC cases using monoclonal antibodies and compared with anatomopathological (AP) variables. The positivity of MRP2 was observed in 92.3% of HCC and in 96.3% of CC. The detection of high MRP2 expression in HCC was not significantly different ($p > 0.05$) according to the size, number of nodules architectural pattern and growth pattern of HCC and CC. Regarding histological grades, 22/22 well moderately differentiated HCC versus 50/56 poorly differentiated HCC were positive for MRP2. A trend for lower expression in poor differentiation HCC was found. And 50/50 well/moderately differentiated CC versus 2/4 poorly/undifferentiated CC were positive for MRP2. This result showed a reduced expression ($p = 0{,}0004$) in poorly differentiated CC. MRP3 positivity was observed in 18.8% of HCC and was not significantly different according to AP parameters. MRP3 was expressed in 44.5% CC, with a trend for lower expression in less differentiated CC and significantly lower rates in the ductular histological subtype ($p = 0.023$). The high expression of MRP2 in HCC and in CC is conserved regardless most of the anatomopathological parameters, except for a trend of lower expression in less differentiated HCC and CC. The observation of lower MRP3 expression in less differentiated CC and, especially, in the histological subtype with expression of hepatic progenitor cell phenotypes leads to future opportunities to evaluate the expression of this marker in cholangiocarcinomas.

Keywords ATP-binding cassette transporters · Hepatocellular carcinoma · Cholangiocarcinoma · Immunohistochemistry

Introduction

The superfamily ABC (ATP Binding Cassette) consists of transmembrane proteins capable of carrying different compounds such as bile salts, phospholipids, ions and anions. Currently, 49 transporter proteins from this family are known and divided into 7 subfamilies: ABCA, ABCB, ABCC, ABCD, ABCE, ABCF and ABCG [1]. Twelve carriers are involved in alterations of homeostasis and human diseases [2, 3]. Recent studies have evaluated the expression of BSEP and MDR3 transporters in hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC), finding promising results regarding the use of these markers in the determination of the cellular lineage of primary hepatic neoplasms, thus potentially useful in the differential diagnosis especially in tissue samples of poorly differentiated neoplasms [4–6]. These initial results raise the demand for further studies of the expression of ABC transporter proteins normally expressed in hepatocytes and cholangiocytes, especially in the more frequent primary malignant neoplasms of the liver, HCC and CC.

✉ Cinthya Santos Cirqueira
cinthyacirqueira@yahoo.com.br

[1] Núcleo de Anatomia Patológica, Centro de Patologia, Instituto Adolfo Lutz, São Paulo, Brazil

[2] Departamento de Patologia, Laboratório de Investigação Médica LIM-14, Universidade de São Paulo, São Paulo, Brazil

Multidrug resistance-associated protein 2 (MRP2) is a multispecific organic anion carrier expressed predominantly in the biliary canaliculum membrane of hepatocytes, gallbladder epithelial cells and the apical membrane of the proximal tubules of normal human kidneys, in the membrane of human cells of the gastrointestinal tract and blood-brain barrier cells [7, 8]. The ABCC2 gene located on human chromosome 10 (10q24) [9, 10] encodes the 1545 amino acid MRP2 protein [11] It is responsible for the excretion of a wide variety of bivalent organic anions related to bilirubin metabolism as conjugated to glutathione, glucoronates and sulfates. It also has affinity to carry leukotrienes, conjugates of bile salts and the metabolism of estradiol.

During transport of glutathione conjugates, this carrier may also export neutral or cationic lipophilic xenobiotics which are related to therapeutic resistance as drugs, carcinogens and toxins, especially when there is presence of overexpression of the gene [7, 12, 13].

Currently, more than 20 hereditary MRP2 gene mutations that result in the absence of the functionally active protein are identified and lead to Dubin-Johnson syndrome characterized by moderate chronic conjugated hyperbilirubinemia [14, 15].

Multidrug resistance-associated protein 3 (MRP3) is present, under physiological conditions, in the basolateral membrane of hepatocytes and cholangiocytes, gallbladder and pancreatic duct cells, as well as present in the adrenal gland, kidney, placenta, stomach and colon [16–19]. The protein consists of 1527 amino acids and approximately 170 kDa molecular weight and it is encoded by the ABCC3 gene located on chromosome 17 (17q21) [20]. Its main function is related to the transport of bivalent (glucuronic and sulfated) conjugated organic compounds and bile salts in hepatocytes and enterocytes [21, 22]. Furthermore, under cholestatic conditions, MRP3 may act as an alternative for the extrusion of bile compounds through the basolateral membrane of hepatocytes in Dubin-Johnson Syndrome in which there is a decrease or complete absence of MRP-2 [23]. Although it has low affinity for amphipathic anions and therefore, limited characteristics related to drug resistance [24, 25], the MRP3 transporter demonstrates to be related to the phenomenon of resistance to methotrexate, vincristine, teniposide and ectoposide [16, 26]. Recently, the overexpression of MRP3 transporter was detected in HCC cell lines resistant to the Sorafenib thyrosin-kinase inhibitor used for the treatment of advanced HCC [27].

In the light of the most recent knowledge on the physiological and pathophysiological role of bile transporter proteins in hepatic metabolism and on the recent demonstration of monoclonal antibodies yielding precise identification of its location in formalin-fixed paraffin embedded tissue samples, the present study aims at the detection of the expression of MRP2 and MRP3 transporters in relatively large series of HCC and CC samples evaluating their potential in the differential diagnosis of these two hepatic neoplasms. Moreover, by comparing this expression with several anatomopathological aspects, our study may generate preliminary subsidies that may serve as a basis for possible future application in therapeutic trials of antineoplastic drugs whose metabolism requires the participation of bile transporter proteins.

Materials and Methods

This study was approved as a research project by hospital's Ethics Committee for Research Project Analysis (CAPPesq) of the University of São Paulo School of Medicine (HC-FMUSP), under the research protocol 368.063 and is centered in a set of 80 sequential autopsy cases of advanced HCC of patients attended at HC-FMUSP (São Paulo/Brazil) between 2003 and 2009 and a set of 56 sequentially cases of surgically resected CC in patients attended at the same hospital between 1992 and 2011.

The anatomicopathological variables of HCC for this study were: number of nodules, size of major lesion, tumor differentiation grade, predominant architectural pattern and presence of cirrhosis. The AP aspects of CC assessed herein were: anatomical location, tumor size, macroscopic growth pattern, histological subtype and tumor differentiation grade.

The slides were reviewed by experienced pathologists (AS, LM, ER, EM, VA) to confirm the diagnosis and classification of anatomopathological variables in all HCC and CC cases. The best preserved and most representative areas of each neoplasm samples fixed in buffered saline formaldehyde pH 7.4 and embedded in paraffin were selected for tissue microarrays (TMA) with spots of 1.0 mm in diameter, with at least two spots per neoplasm. In cases with more than one neoplastic nodule, the largest nodule was selected to represent that patient in the comparisons with clinical-pathological findings.

Immunohistochemical reactions were performed manually with epitope retrieval in a steamer (Tris-EDTA pH 9 1 mM solution), followed by overnight incubation with primary antibodies anti-MRP2 (monoclonal M2 III-6, Abcam ab3373, dilution 1: 600) and anti-MRP3 (monoclonal DTX −1, Abcam ab49479, dilution 1: 400). Signal amplification was achieved through the polymer conjugated with peroxidase (Novolink, Novocastra/Leica Biosystems).

Expression levels were evaluated semiquantitatively for the intensity (0, 1+, 2+, 3+ and 4+) and for the frequency of positive cells (on decimal scales: 0–10%, >10–20%, > 20–30% ... > 90 to 100%). The intensity value of each spot of the case was multiplied by the frequency of positive cells (0 to 100%), generating a score between 0 and 400. The highest score obtained among all spots of each node ("hot spots") was chosen as the valid score to represent that node. Cases with a score greater than 10 were considered positive, i.e. 20 or more (2 × 10 or 1 × 20 or more).

The comparison between the positivity score of the immunohistochemical reactions for each antibody and the clinical-pathological variables was performed using chi-square or Fisher's Exact tests, establishing $p < 0.05$ as the level of statistical significance.

Results

Clinical and Anatomopathological Data

From the 80 cases of HCC studied herein, 62 (78.5%) were male patients and 18 (22.5%) female. The patients' ages ranged from 28 to 82 years, with a mean of 58.1 ± 10.9 (mean ± SD) years and a median of 59.5 years.

The main cause of HCC was HCV infection in 33.7% (27/80) of cases followed by alcoholism in 13.8% (11/80) and HBV infection in 11.2% (9/80) of the cases. In 15/80 (18.8%) cases, combinations were detected among these factors. We also identified 2 (2.5%) cases of HCC occurring in cirrhosis due to hemochromatosis. In 10 (12.5%) cases, the etiological investigations did not lead to the identification of the cause (cryptogenic) and another 6 (7.5%) cases had no data available.

The anatomopathological examination identified cirrhosis in 72 (90%) cases. Vascular invasion was detected in 15 (19%) cases. In 43 (54%) of the cases, 4 or more tumor nodules were detected, 1 (1%) cases with 3 nodules, 4 (5%) cases with 2 nodules and 24 (30%) cases with a single tumor nodule. The size of the largest tumor nodule was identified in the original macroscopic report in 59 (73.8%) cases and ranged from 0.8 to 18.0 cm with a mean of 5.4 ± 4.1 (mean ± SD) and median of 4.1 cm.

From the 80 cases of HCC, the predominant architectural pattern was trabecular in 36 (45%) cases, followed by 24 cases (30%) of solid/macrotrabecular pattern, 9 (11%) of acinar/pseudoglandular pattern and 9 (11%) of mixed pattern. Two cases (3%) presented the clear cell variant. Our review identified HCC Edmondson-Steiner grade I in 1 case (1.2%), 21 (26.3%) grade II, 47 (58.8%) grade III and 11 (13.7%) grade IV.

The original anatomopathological report of the 56 CC cases yielded information of the gender of patients in 52 cases (92.9%) presented information on the sex of the patients, 19 (36.5%) female and 33 (63.5%) male. The mean age in 47 patients identified in the exam was 56 ± 13.2 (mean ± SD) and median age of 57 years, ranging from 23 to 86 years.

The site distribution of the CC was intrahepatic in 19.7% (11/56) cases, 32.1% (18/56) cases of extrahepatic cholangiocarcinoma and 48.2% (27/56) cases of hilar cholangiocarcinoma. Tumor sizes were reported in 49 (83.9%) cases and varied between 1.5 and 23 cm with a mean of 10.7 ± 6.3 (mean ± SD) and a median of 12.0 cm. The macroscopic growth pattern of the CC was mass-forming in 20 (35.7%) cases, periductal infiltrative type in 35 (62.5%) cases and intraductal type in 1 (1.8%) case. The presence of vascular invasion was observed in 22.9% (11/48) of the cases, perineural invasion in 62.3% (33/53) of the cases and metastases in 17% (9/53) of the cases.

Microscopic examination showed that 76.8% (43/56) cases were classified as "ductal", 7.1% (4/56) cases of ductular malformation plate and 16.1% (9/56) cases of the ductular type of intermediate cells. Thirty-three (58.9%) cases were classified as well differentiated, 19 (33.9%) moderately differentiated, 3 (5.4%) cases were poorly differentiated and 1 case (1,8%) undifferentiated.

Anatomopathological information of the biliary tract neoplasms and the grouping of the cases for statistical purposes are summarized in Table 1 and Table 2.

Expression of MRP2 - Multidrug Resistance-Associated Protein 2

The reaction was valid in 78/80 cases (97.5%) of HCC present in TMA, since only 2 cases were lost in histological and immunohistochemical processing. The anti-MRP2 antibody recognized an epitope present in the canalicular region and on the basolateral face of normal and neoplastic hepatocytes, sometimes assuming a circumferential pattern, with varying intensity. The linear, acinar (pseudoacinar) and punctate expression patterns were frequently detected in the samples evaluated (Fig. 1A and B).

The reaction was valid in 54/56 cases (96.4%) of CC. Reactivity for MRP2 was observed in the lateral and luminal membranes of cholangiocytes in normal, proliferated in ductular reaction and in neoplastic cells. The expression pattern exhibited uniform and sometimes granular linear labeling with intensity and distribution ranging from moderate to intense (Fig. 1C and D).

The reaction for MRP2 protein was positive in 92.3% (72/78) of HCC cases and in 52/54 cases (96.3%) of CC. The association between the IHC reactivity of MRP2 and the AP variables of HCC and CC is depicted at Tables 3 and 4. The high MRP2 expression was found independent of all anatomopathological aspects of HCC and CC, except for histological grading: although not statistically significant, all 22 well/moderately differentiated HCC were positive for MRP2 versus only 50/56 poorly differentiated HCC. MRP 2 was positive in all 50 well/moderately differentiated cases but in only 2/4 poorly/undifferentiated cases. There was a significant reduction ($p = 0{,}00042$) of MRP2 expression in poorly differentiated cases (Tables 3 and 4).

Expression of MRP3 - Multidrug Resistance-Associated Protein 3

The reaction was valid in all 80 (100%) cases of HCC. The antibody recognized an epitope present on the basolateral membrane of normal and neoplastic hepatocytes with variable

Table 1 Summary of anatomopathological aspects in 80 autopsies with hepatocellular carcinoma

Features		n (%)
Number of nodules	1	24 (30,0)
	2	04 (5,0)
	3	01 (1,0)
	4 or more	43 (54,0)
	Unknown	08 (10,0)
Tumor size (cm)	Mean ± S.D.	5,4 ± 4,1
	Medium	4,1
	Minimum - maximum	0,8–18,0
Predominant histological pattern or variant	Trabecular	36 (45,0)
	Acinar/pseudoglandular	09 (11,0)
	Solid/macrotrabecular	24 (30,0)
	Mixed	09 (11,0)
	Clear cells	02 (3,0)
Tumor differentiation grade (Edmondson-Steiner)	I + II	22 (27,5)
	III	47 (58,8)
	IV	11 (13,7)

intensity, depicting linear and acinar expression patterns (Fig. 2E and F). On the other hand, in CC, the reaction was valid in 54/56 cases (96.4%). The antibody recognized an epitope present on the lateral membrane of normal and neoplastic cholangiocytes with variable intensity. Reactivity was also identified on the luminal face of the normal and proliferated bile duct epithelium (ductular reaction) with variable intensity (Fig. 2G and H).

The immunohistochemical investigation of the MRP3 protein was positive in only 15/80 (18.8%) cases of HCC. The low expression of MRP3 in HCC was found independent of the anatomopathological parameters (Tables 3 and 4).

Regarding cholangiocarcinoma, 24/54 cases (44.5%) were found positive for MRP3, with no significant variation between the positivity score for MRP3 and AP variables of topography and growth pattern of cholangiocarcinoma cases. Regarding histological grading, although not achieving significant *p*-values, a trend for a different frequency of MRP3 expression was found: 24/50 well/moderately differentiated CC were found positive for MRP3, versus 0/4 poorly/undifferentiated CC cases. Still more relevant, the analysis of reactivity for MRP3 by histological type demonstrated a significantly lower MRP3 expression in cases of ductular histological subtype (plate malformation and intermediate cell) ($p = 0.023$).

Table 2 Summary of anatomopathological aspects in 56 cases of cholangiocarcinoma

Features		n (%)
Anatomical location	Intrahepatic peripheral	11 (19,7)
($n = 56$)	Hilar and extrahepatic	45 (80,3)
Tumor size (cm)	Mean ± S.D.	
($n = 49$)		10,7 ± 6,3
	Medium	12
	Minimum - maximum	1,5–23
Tumor size	< 12,0 cm	23
($n = 49$)	≥12,0 cm	26
Macroscopic pattern (n = 56)	mass-forming	20 (35,7)
	Infiltrative periductal and Intraductal	36 (64,3)
Histological subtype	Ductal	43 (76,8)
($n = 56$)	Ductular malformation plate and Ductular of intermediate cells	13 (23,2)
Tumor differentiation grade	Well differentiated	33 (58,9)
($n = 56$)	Moderately differentiated, Poorly differentiated and undifferentiated	23 (41,1)

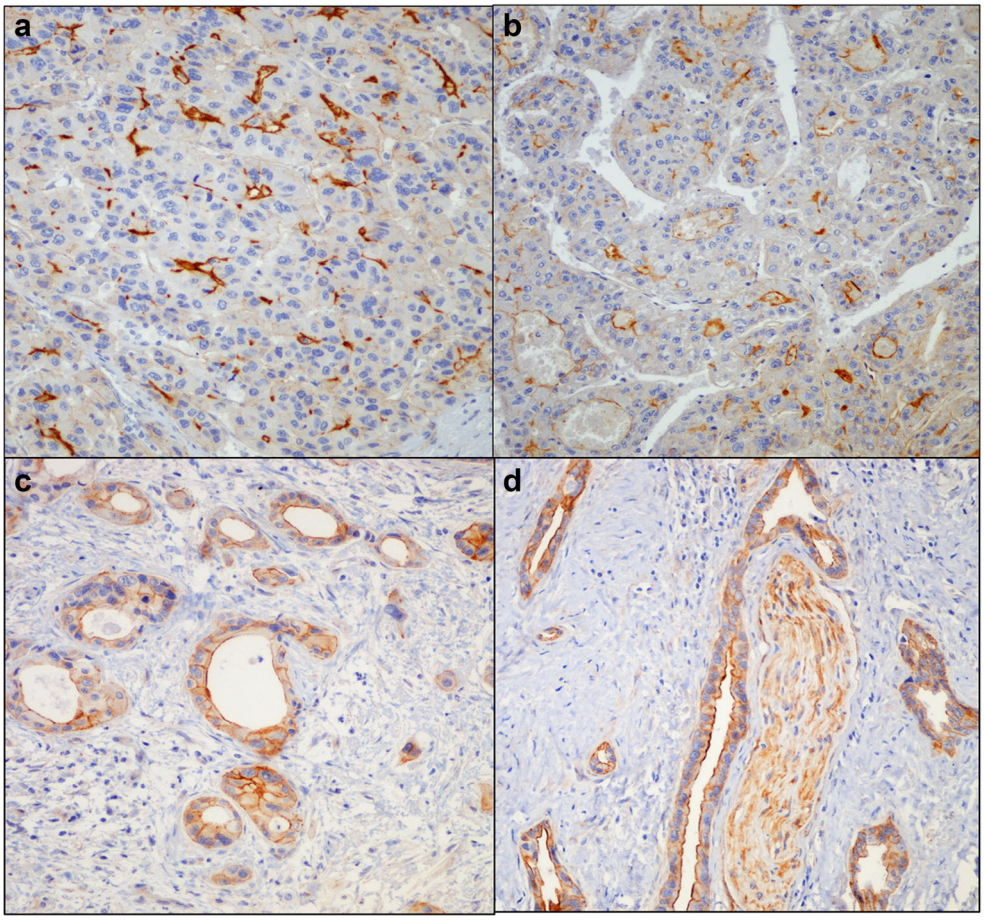

Fig. 1 IHC Reactivity of MRP2. Positivity observed in the canalicular membrane with linear and acinar (pseudoglandular) expression pattern in hepatocellular carcinoma (A, B: 200X) and luminal and lateral expression pattern in cholangiocarcinoma (C, D: 200X)

Table 3 Association between reactivity for MRP2 and MRP3 and anatomopathological variables of hepatocellular carcinoma

Anatomopathological Aspects	MRP2			MRP3		
	Negative n (%)	Positive	P	Negative n (%)	Positive	P
Number of nodules						
1	4 (16,7)	20 (83,3)	0,171	16 (66,7)	8 (33,3)	0,121
> 1	2 (4,3)	44 (95,7)		41 (85,4)	7 (14,6)	
Tumor size						
≤ 2.0 cm	0 (0,0)	13 (100,0)	0,552	12 (80,0)	3 (20,0)	0,999
> 2.1 cm and ≤ 5.0 cm	2 (9,5)	19 (90,5)		16 (76,2)	5 (23,8)	
> 5.1 cm	2 (8,7)	21 (91,3)		18 (78,3)	5 (21,7)	
Architectural Pattern						
Trabecular	4 (11,1)	32 (88,9)	0,453	30 (81,1)	7 (18,9)	0,079
Pseudoglandular	0 (0,0)	9 (100,0)		7 (77,8)	2 (22,2)	
Solid	1 (4,3)	22 (95,7)		22 (91,7)	2 (8,3)	
Mixed	0 (0,0)	8 (100,0)		4 (50,0)	4 (50,0)	
Tumor differentiation grade						
I	0 (0,0)	1 (100,0)	0,176	1 (100,0)	0 (0,0)	0,334
II	0 (0,0)	21 (100,0)		15 (71,4)	6 (28,6)	
III	5 (11,2)	40 (88,8)		39 (83,0)	8 (17,0)	
IV	1 (9,1)	10 (90,9)		10 (90,9)	1 (9,1)	

Table 4 Association between reactivity for MRP2 and MRP3 and anatomopathological variables of cholangiocarcinoma

Anatomopathological Aspects	MRP2			MRP3		
	Negative n (%)	Positive	P	Negative n (%)	Positive	P
Anatomical location						
Peripheral intrahepatic	1 (9,1)	10 (90,9)	0,369	8 (72,8)	3 (27,2)	0,310
Hilar	0 (0,0)	26 (100,0)		13 (50,0)	13 (50,0)	
Extrahepatic	1 (5,9)	16 (94,1)		9 (53,0)	8 (47,0)	
Growth pattern						
mass-forming	1 (5,0)	19 (95,0)	1000	14 (70,0)	6 (30,0)	0,156
Periductal	1 (3,0)	32 (97,0)		15 (45,5)	18 (55,5)	
Intraductal	0 (0,0)	1 (100,0)		1 (100,0)	0 (0,0)	
Histological subtype						
Ductal	1 (2,5)	40 (97,5)	0,427	19 (46,3)	22 (53,6)	0,023
Ductular malformation plate	0 (0,0)	4 (100,0)		3 (75,0)	1 (25,0)	
Ductular intermediate cells	1 (11,1)	8 (88,9)		8 (88,9)	1 (11,1)	
Tumor differentiation grade						
Well differentiated	0 (0,0)	31 (100,0)	0,0004	14 (45,2)	17 (54,8)	0,099
Moderately differentiated	0 (0,0)	19 (100,0)		12 (63,1)	7 (36,9)	
Poorly differentiated	1 (33,3)	2 (66,7)		3 (100,0)	0 (0,0)	
Undifferentiated	1 (100,0)	0 (0,0)		1 (100,0)	0 (0,0)	

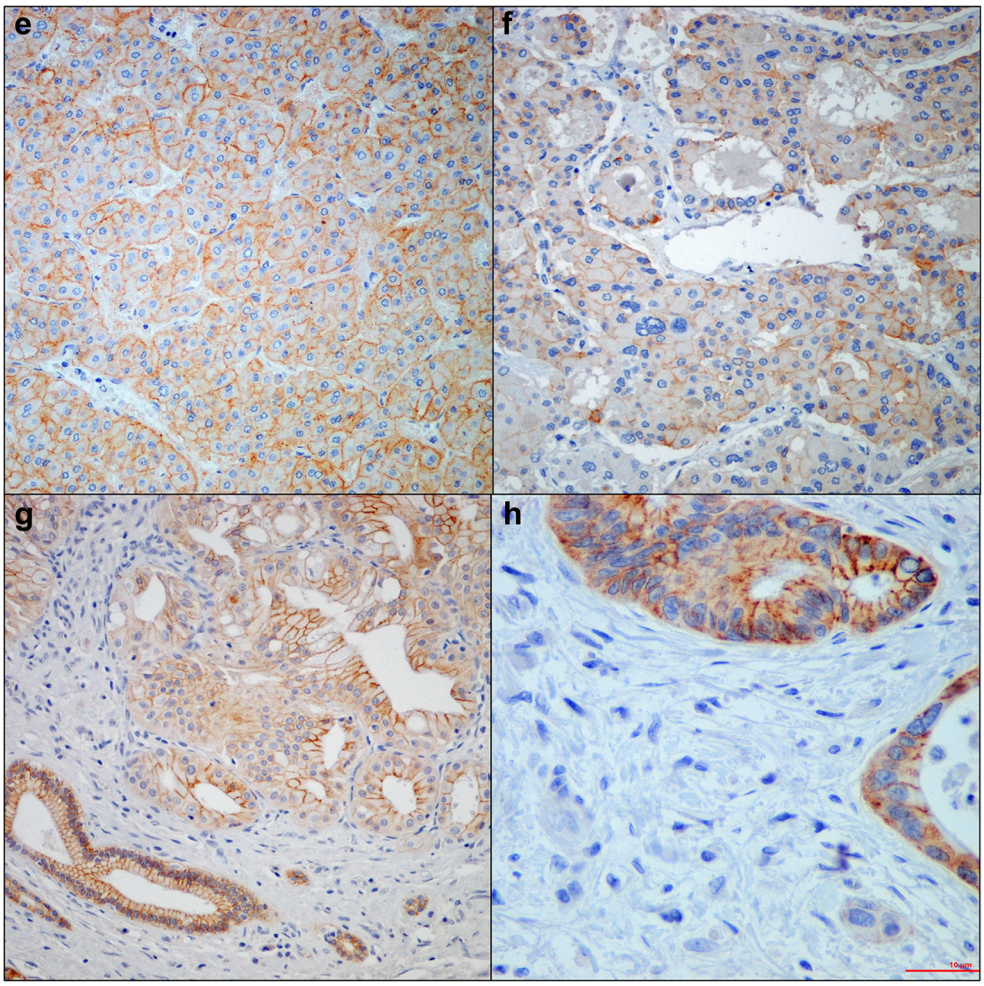

Fig. 2 MRP3 IHC Reactivity. Positivity observed in the basolateral membrane in hepatocellular carcinoma (E, F: 200X) and basolateral and luminal in cholangiocarcinoma (G, H: 200X)

Discussion and Conclusions

The present study demonstrated the efficacy of the anti-MRP2 M2 III-6 monoclonal antibody and the anti-MRP3 monoclonal antibody DTX1 in the detection of these bile transporter proteins in formalin-fixed paraffin-embedded HCC, allowing their precise localization in cell membranes.

MRP2 was found positive in 72/80 cases (92.3%) of HCC. Different membrane patterns (linear, punctate and acinar) were observed simultaneously in cases of HCC. The acinar pattern identified in our study had also been recognized in cells with pseudoglandular arrangement in other studies [28, 29].

The absence of significant variation of MRP2 expression in relation to number, nodule size, architectural standard (including solids), suggests that the MRP2 protein is highly expressed in HCC independently of the anatomopathological variables analyzed. Further studies based on larger representation of poorly differentiated HCC might be useful to assess a possible trend for lower rates of expression of MRP2 in these less differentiated cases. MRP2 was also found positive in 96.3% (52/54) of cases of CC and the expression pattern exhibited linear and sometimes granular marking, with intensity and distribution varying from moderate to severe in the lateral membranes and in luminal aspect of normal, proliferated and neoplastic ducts.

MRP2 expression in more than 90% of the malignant neoplasms of both hepatic epithelium do not favor the use of this marker for the differential diagnosis between HCC and CC, in agreement with the relatively few available studies reporting such comparison [28–33]. On the other hand, such frequent positivity and reactivity patterns as well characterized in the canalicular pole (apical pattern), in the basolateral or even circumferential face, open the perspective of future assessment of possible relation of these different patterns of MRP2 expression with aspects of the pathogenesis, or even with the metabolism of therapeutic agents for these neoplasms.

Our bibliographic research allowed us to identify the study by Rau et al. [26] who investigated the expression of MRP2 and MRP3 in cultured cells and samples of paraffin-shaped tissue from cholangiocarcinomas, gallbladder adenocarcinomas by immunofluorescence. Those investigators used the polyclonal EAG antibody and reported low expression (29%) of MRP2 in gallbladder neoplasia and absence in 7 cases of extrahepatic cholangiocarcinoma (5 cases grade 2 and 2 cases grade 3), and were therefore different from our present results. Such discrepancy may result from our use of M2 III-6 monoclonal antibody and from the detection system amplification with secondary antibodies conjugated to short polymers labeled with peroxidase molecules.

The high sensitivity for this marker was observed in our sample, composed of 54 cases of CC, in all anatomopathological parameters analyzed, which included different locations (intra and extrahepatic), types of growth pattern and, histological subtypes (ductal and ductular). Regarding MRP2 expression according to histological grade of differentiation in CC, MRP2 was found positive in all 50 well/moderately differentiated cases, whereas only 2/4 poorly / undifferentiated. CC case was positive for MRP2.

Therefore, the scarcity of information and the presence of divergent results on the evaluation of the expression of this protein in cholangiocarcinoma require further studies to assess the frequency and the pattern of expression of this carrier in a larger population of intrahepatic, hilar and extrahepatic cholangiocarcinomas.

MRP3, another functionally important protein in biliary transport in hepatocytes and in bile ducts, in the present study, was expressed in only 15 (18.8%) of the 80 HCC cases showing basolateral distribution in more than 10% of tumor hepatocytes. These our findings, aligned to those of Borght [30] suggest little use of MRP3 as a marker of diagnostic interest in HCC. However, the excellent contrast between the positive signal and negative background tissue, yielding the identification of different distribution of positivity patterns in each case may provide other applications for the detection of the important bile transporter MRP3 in the study of hepatic neoplasms. Already in 2001, Nies et al. [29] pointed to evidence of a possible association of tissue positivity to MRP3 with chemoresistance, finding, through immunofluorescence reaction, basolateral membrane positivity in all 9 cases of well or moderately differentiated HCC frozen samples.

Zollner et al. [28] assessed the potential of detection of carrier proteins, including MRP3, as predictor of chemoresistance in frozen hepatocellular carcinoma samples. Those authors observed that 75% (3/4) of the evaluated cases were found negative for MRP3, whereas the cytoplasmic reactivity in one case was explained by a protein migration disorder.

Reinforcing the hypothesis that the detection of MRP3 overexpression might be related to mechanisms of resistance to tyrosine kinase inhibitors related to HCC treatment, Tomonari et al. [27] point out that MRP3 may prove to be a resistance factor to the therapeutic action of Sorafenib. Based on those preliminary data raised by Nies et al., Zollner et al. and Tomonari et al. and due to the precise identification of different membrane patterns of membrane staining found in our cases, we would suggest that therapeutic trials in the near future should test the predictive value of the expression of these markers in the selection of patients for treatment with such kinase inhibitors.

Our study found that MRP3 was positive in 44.5% (24/54) of cholangiocarcinoma cases. The only similar study found in the literature was performed by Rau et al. [26] in 2008 for the evaluation of resistance to chemotherapy treatment. Those authors observed immunoexpression of MRP3 by immunohistochemistry in 7 samples of extrahepatic cholangiocarcinoma (5 moderately differentiated and 2 poorly differentiated) and in 14 gallbladder carcinomas. They reported positivity for MRP3 in 57% (4/7) of cases of extrahepatic cholangiocarcinoma.

We also evaluated the expression of MRP3 in relation to anatomopathological parameters: no difference of MRP3 expression was herein found regarding topography and macroscopic growth patterns. However, although only 4 CC cases presented poorly/undifferentiation, these cases tended to present lower expression of MRP3 than the better differentiated cases, requiring further studies in a larger population of CC, especially in intrahepatic CC cases. Perhaps still more relevant, a significantly lower expression of MRP3 was found in the ductular histological subtypes (2/13 cases versus 22/41 cases of larger duct subtype). This presentation, which includes the intermediate cell pattern and ductal plate malformation) resembles bile duct proliferation and expression of hepatic progenitor cell phenotypes [34, 35]. This finding opens the opportunity for future studies seeking to relate such positivity to other clinical and histological parameters of cholangiocarcinomas, especially for intrahepatic cholangiocarcinoma whose patterns of expression of MRP3 is, yet, largely unknown.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest with respect to this article.

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