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ORIGINAL ARTICLE

Prognostic role of BAP-1 and PBRM-1 expression in intrahepatic cholangiocarcinoma

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Abstract

Intrahepatic cholangiocarcinoma (ICC) has universally poor outcome, mainly due to its late clinical presentation. Identification of specific biomarkers and development of effective treatment are still urgently required. Mutations in *PBRM-1* and *BAP-1* genes, and the expression of S100P have been related to survival in ICC. miR-31 seems also to play important regulatory functions in ICC and it directly regulates BAP-1 expression in lung cancer. In this study, tissue expression of BAP-1, PBRM-1, S100P, and miR-31 was investigated in ICC and correlated with clinical-pathological features. Sixty-one consecutive patients who underwent curative hepatic resection for ICC were enrolled. None received any therapy prior to surgery. Immunostaining for BAP-1, PBRM-1, and S100P, and in situ hybridization for miR-31 were performed, using tissue microarray slides. A strong retained expression of BAP-1 and PBRM-1 was associated with a reduced overall ($p = 0.04$ and $p = 0.002$, respectively) and disease-free survival ($p = 0.05$ and $p = 0.02$, respectively). An overexpression of S100P was related to a reduced overall survival ($p = 0.005$). The multivariate analyses identified the presence of perineural invasion and the retained PBRM-1 expression as independent predictors of worse overall [$p = 0.02$, hazard ratio (HR) = 2.25 (1.16–4.39) and $p = 0.001$, HR = 3.13 (1.56–6.28), respectively] and disease-free survivals [$p = 0.03$, HR = 2.43 (1.09–5.4) and $p = 0.03$, HR = 2.51 (1.11–5.67), respectively]. An overexpression of S100P was predictive of a worse overall survival [$p = 0.02$, HR = 1.66 (1.08–2.55)]. High levels of miR-31 were significantly associated to a low expression of BAP-1 protein ($p = 0.03$). In ICC, a retained expression of BAP-1 and PBRM-1, and an overexpression of S100P are related to a poor prognosis.

Keywords Intrahepatic cholangiocarcinoma · BAP-1 · PBRM-1 · S100P · Prognostic role · miR-31

Introduction

Intrahepatic cholangiocarcinoma (ICC) is the second most common primary liver tumor, with an increasing incidence over the past decades, particularly in Western countries [1–3]. Prognosis of ICC is generally poor, and it remains a challenging disease in terms of diagnosis and treatment [1, 4]. In fact, due to the lack of early symptoms and specific biomarkers, ICC diagnosis is frequently reached at advanced stages, when treatment options are very limited [2]. Nowadays, surgery remains the only potentially curative treatment, but outcome remains dismal even after radical resection, with high recurrence rates [1–4].

Several genetic and epigenetic studies have defined ICC as a molecularly heterogeneous malignancy [3, 5–8].

It has been reported that genes usually acting as tumor suppressor genes and involved in chromatin remodeling, such as *BRCA1-associated protein-1* (*BAP-1*) and *polybromo-1* (*PBRM-1*), are frequently mutated in ICC [5–13]. In particular, somatic mutations in these genes are related to a worse overall survival and an aggressive metastatic phenotype, and then to an adverse prognosis [3, 5–8]. Immunohistochemical (IHC) evaluation of the BAP-1 protein is used in the diagnosis and prognostic stratification of patients with various cancer

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types, including mesothelioma and melanoma [11, 14]. As for ICC, a single study was able to demonstrate that the complete loss of BAP-1 expression was related to favorable clinical-pathological features and to a better prognosis [15], suggesting a potential use of this biomarker in the prognostic evaluation of ICC patients. The same authors reported that the loss of PBRM-1 expression was not associated with any specific prognostic or genetic characteristic instead [13, 15].

MiRNAs also seem to play important regulatory functions in ICC [3, 6], and it was shown that an upregulation of miR-31 may promote oncogenesis in this tumor [6, 16]. Interestingly, as previously reported, miR-31 is a direct regulator of BAP-1 expression in lung cancer, and its overexpression contributes to disease progression [17].

S100P is a member of the S100 family of calcium-binding proteins with a diagnostic potential in ICC [18, 19]. Its expression in this tumor type has been suggested to have a prognostic value as well, being related to an aggressive behavior and poor prognosis [20, 21]. Moreover, previous IHC studies suggested that S100P is able to identify a subtype of ICC that may develop from large bile ducts [21, 22].

Within this background, the aim of our study was to investigate the tissue expression and the clinical-pathological correlations of BAP-1, PBRM-1, S100P, and miR-31 in ICC.

Materials and methods

Case selection

A total of 78 consecutive patients with a diagnosis of primary ICC, who underwent laparoscopic hepatic resection with curative intent from January 2006 to December 2014 at the University Hospital of Padova, were retrospectively considered. All the patients gave their appropriate informed consent to the procedure. The exclusion criteria were i) the administration of any therapy prior to surgery, both systemic or locoregional, to avoid any bias affecting survival analysis; ii) a survival of less than 3 months after surgery, to exclude deaths due to surgical complications; and iii) the absence of available tissue for further IHC stains. Only cases with a histological diagnosis of conventional type ICC according to Nakanuma et al. [22] were included. Sixty-one tumors were finally selected for the study. The study complies with the ethical guidelines of the 1975 Declaration of Helsinki and obtained the approval from the local Ethics Committee (Ethics Committee for Clinical Research—University Hospital of Padova, Italy; protocol #: 0038038/2017).

Clinical data

All relevant patients' clinical and laboratory data were retrieved from medical charts, including sex, age, serum levels of carcinoembryonic antigen (CEA) and CA 19–9 at the time of surgery, the presence of any underlying chronic hepatic or biliary disease, the presence of cirrhosis, and whether the patients underwent adjuvant chemotherapy or not.

All of the patients were clinically followed-up, and physical examination, serum tumor markers levels, ultrasonography, and computed tomography were regularly performed to detect recurrence. Overall and disease-free survival time was obtained from medical records.

Histological study

All of the cases were blindly and contemporarily reviewed by an experienced (MG) and a trainee (SS) liver pathologist, and histologically classified according to Nakanuma et al. [22]. The macroscopic tumor type, the grade of differentiation, the T stage (according to the revised 8th edition of the AJCC staging system), and the presence of vascular and perineural invasion and lymph node metastasis were also recorded.

Immunohistochemical study

Tissue microarrays composed of 4-mm cores of formalin-fixed paraffin-embedded tumor tissue were built by selecting two representative tumor areas from each case. All of the samples were processed by using the Galileo CK3500 Arrayer (www.isenet.it), a semi-automatic and computer-assisted TMA platform, as previously described [23].

Immunostaining was performed on tissue microarray sections by using the following antibodies: anti-BAP-1 (Santa Cruz Biotechnology, Dallas, TX, USA; dilution 1:40; mouse monoclonal), anti-PBRM-1 (Bethyl Laboratories Inc., Montgomery, TX, USA; dilution 1:300; rabbit polyclonal), and anti-S100P (Abcam, Cambridge, UK; dilution 1:1000; rabbit monoclonal). IHC staining was conducted according to standard techniques by using the Leica Microsystems Bond-Max autostainer (Leica Biosystems, Newcastle Upon Tyne, UK), and all the slides were counterstained with hematoxylin. Appropriate positive and negative controls were used for each run.

In the evaluation of BAP-1 and PBRM-1, only nuclear staining was considered, while S100P expression was both nuclear and cytoplasmic. BAP-1 and S100P expression was scored on a scale from 0 to 2+: 0 = negative or present in < 1% of tumor cells, 1+ = expression in ≥ 1% but < 90% of tumor cells or patchy expression, and 2+ = diffuse positivity in ≥ 90% of the tumor (Fig. 1). PBRM-1 expression was scored as absent/present (Supplementary Fig. 1). In each case and for each antibody, a completely absent nuclear or cytoplasmic reaction in the neoplastic cells was considered negative only if a positive internal control was present, that is non neoplastic cells such as lymphocytes, normal bile duct cells (as for BAP-

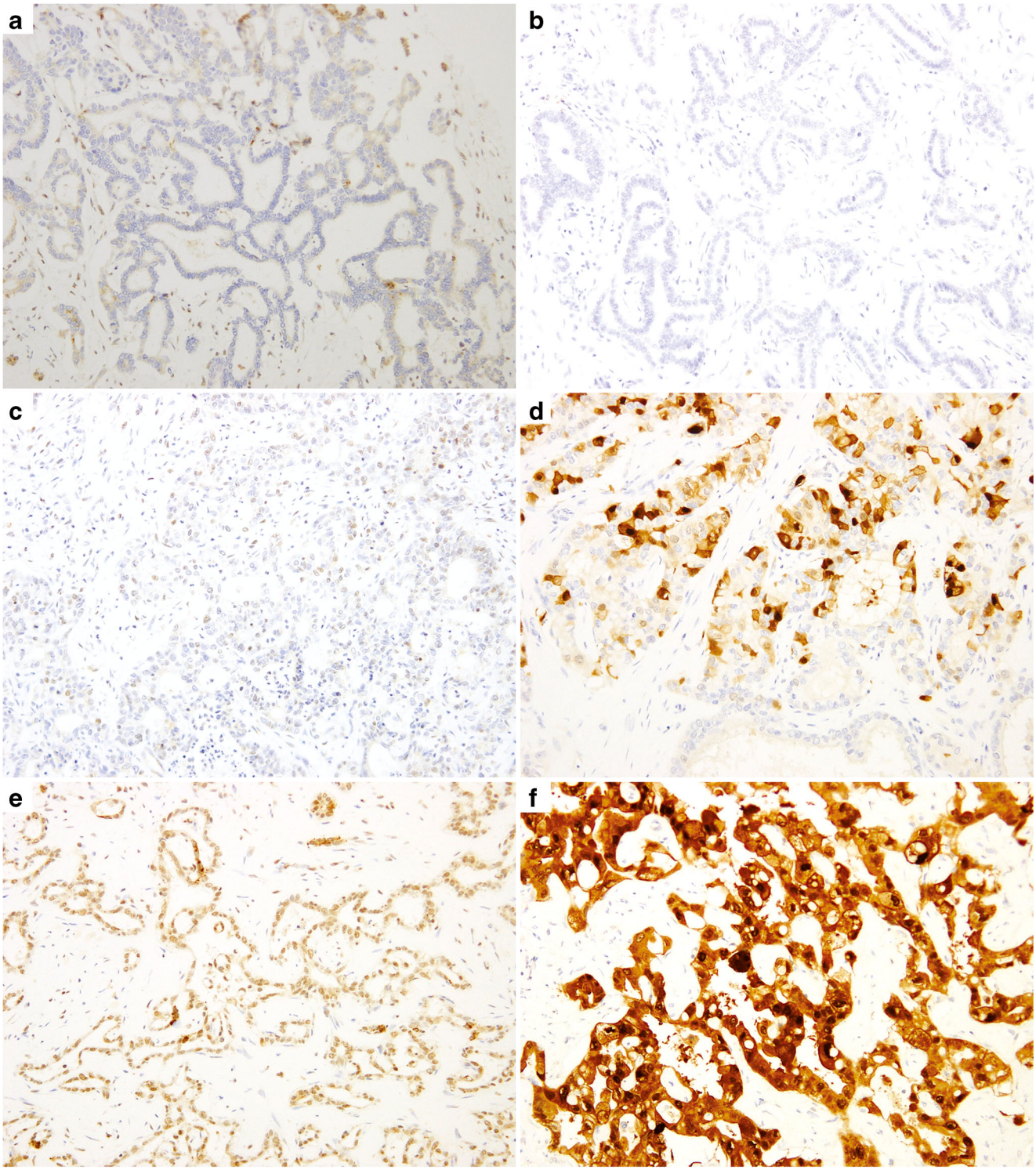

Fig. 1 BAP-1 and S100P expression in cases of intrahepatic cholangiocarcinoma. As reported in the text, a BAP-1 (**a**, original magnification 10×) and S100P (**b**, original magnification 10×) reaction was considered negative only if a positive internal control was present. BAP-1 and S100P expression was scored as 1+ when present in ≥ 1% but < 90% of tumor cells or when a patchy expression was observed (**c**, BAP-1, original magnification 10×; **d**, S100P, original magnification 10×), and 2+ when a diffuse positivity was detected (**e**, BAP-1, original magnification 10×; **f**, S100P, original magnification 10×). Of note, only nuclear BAP-1 staining was considered, while S100P expression was both nuclear and cytoplasmic

1 and PBRM-1) or stromal cells, otherwise the staining was defined inadequate and repeated.

miR-31 in situ hybridization

Locked nucleic acid probes with complementarity to miR-31-5p were labeled with 5′-digoxigenin and synthesized by Exiqon (Copenhagen, Denmark). Tissue sections were digested with in situ hybridization (ISH) protease 1 (Ventana Medical Systems, Milan, Italy) and ISH was performed as described, with minor modifications [24]. Positive (U6; Exiqon, Copenhagen, Denmark) and negative scrambled locked nucleic acid probes were used as controls. Cases were classified according to the cytoplasmic miR-31 intensity as: negative = negative or faint expression in most cells; low expression = mild expression in most cells or moderate expression in < 50% of the cells; high expression = moderate to strong expression in most cells (Fig. 2).

Statistical analysis

Continuous variables were expressed as median (range) and categorical variables as frequency and percentage. For clinical-pathological correlations, Mann–Whitney test (Rank Sum Test), Wilcoxon test, Spearman rank correlation test, and Fisher exact probability test were used when appropriated. Overall and disease-free survival curves were generated by using the Kaplan-Meier method, and compared by using the log-rank and Wilcoxon tests. The multivariate Cox regression analyses were performed through the use of the pathological variables that were identified as significant on univariate survival analyses. Hazard ratios (HRs) and their 95% confidence intervals (CIs) were also calculated. A p value < 0.05 was considered significant. The data analysis was performed by applying SPSS statistical program (version 20.0, IBM SPSS Statistics, Chicago, IL, USA).

Results

The patients were 26 males (42.6%) and 35 females (57.4%), with a median age of 67 years (range 35–82 years) and a median follow-up of 2.2 years (range 0.3–8.8 years). The clinical and laboratory features of the patients are summarized in Table 1. Nine patients (14.8%) had cirrhosis. Lymphadenectomy was performed in only 35/61 cases. Fifteen patients out of 61 underwent

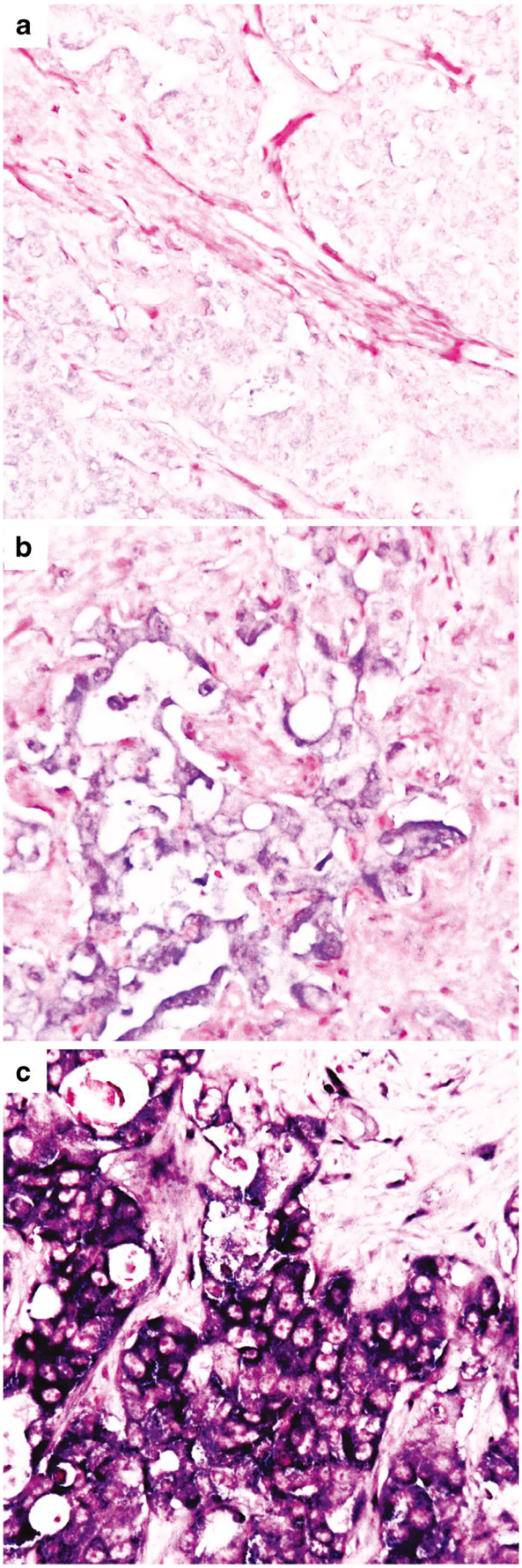

Fig. 2 miR-31 expression in intrahepatic cholangiocarcinoma. As reported in the text, cases were classified, according to the cytoplasmic miR-31 intensity, as negative (**a**, original magnification 60×), with a low (**b**, original magnification 60×) or with a high (**c**, original magnification 60×) miR-31 expression

Table 1 Clinical and laboratory features of the patients

Feature	*N* = 61
Age [years]	
Median (range)	67 (35–82)
Sex	
N (%)	
Males	26 (42.6)
Females	35 (57.4)
CEA [ng/ml]	
Median (range)	2.1 (0.5–114.6)
CA 19.9 [U/ml]	
Median (range)	81.3 (0–16,027)
Underlying diseases	
N (%)	
HBV hepatitis	1 (1.6)
HCV hepatitis	6 (9.8)
Alcoholic hepatitis	1 (1.6)
NAFLD/NASH	2 (3.3)
Cirrhosis	
N (%)	9 (14.8)
Adjuvant chemotherapy	
N (%)	15 (24.6)
Recurrence	
N (%)	30 (49.2)
Exitus	
N (%)	41 (67.2)

CEA, carcinoembryonic antigen; *HBV*, hepatitis B virus; *HCV*, hepatitis C virus; *NAFLD*, non-alcoholic fatty liver disease; *NASH*, non-alcoholic steatohepatitis

adjuvant chemotherapy. Age, sex, and the presence or absence of underlying chronic liver diseases were not related to patients' prognosis. Moreover, there were no differences in the overall and disease-free survivals between patients who underwent adjuvant chemotherapy and those who did not, neither between patients with or without cirrhosis, the latter probably explained by the low number of cirrhotic patients in our cohort.

Histological features and clinical-pathological correlations

All the cases were macroscopically mass forming and histologically classified as conventional ICCs, as set out in the selection criteria. There were 35 cases (57.4%) of small bile duct subtype and 26 (42.6%) of the large bile duct one. Vascular invasion was present in 39/61 patients (63.9%), while perineural invasion was observed in 27/61 cases (44.3%), as reported in Table 2. Lymph node metastases were detected in 11 patients out of 35 who underwent lymphadenectomy. Twenty-four out of 61 patients (39.3%) had a microscopic neoplastic infiltration of the resection margin (R1 cases), while the remaining 37 patients underwent a complete surgical resection of the tumor (R0 cases).

Table 2 Histological and immunohistochemical data of the cases

Feature	*N* = 61
Histological subtype	
N (%)	
Small bile duct	35 (57.4)
Large bile duct	26 (42.6)
Grade	
N (%)	
G1	2 (3.3)
G2	28 (45.9)
G3	31 (50.8)
T stage	
N (%)	
T1(a–b)	17 (27.9)
T2	28 (45.9)
T3	14 (22.9)
T4	2 (3.3)
Vascular invasion	
N (%)	39 (63.9)
Perineural invasion	
N (%)	27 (44.3)
Lymph node metastasis	(*N* = 35)
N (%)	11 (31.4)
BAP-1	
N (%)	
0	22 (36.1)
1+	16 (26.2)
2+	23 (37.7)
PBRM-1	
N (%)	
Absent	26 (42.6)
Present	35 (57.4)
S100P	
N (%)	
0	30 (49.2)
1+	19 (31.1)
2+	12 (19.7)
miR-31	
N (%)	
Negative	1 (1.6)
Mild expression	37 (60.7)
High expression	23 (37.7)

As expected, both perineural and vascular neoplastic invasion were related to a worse overall ($p = 0.0009$ and $p = 0.04$, respectively) and disease-free survival ($p = 0.02$ and $p = 0.0009$, respectively). An advanced T stage (i.e., T stage 3 and 4) was related to a reduced overall survival ($p = 0.02$). We did not find any difference in the survival between the

small and the large bile duct subtypes of ICC, nor between R0 and R1 cases. We also failed to detect a significantly different survival in patients with lymph node metastases, but this could be due to the low number of performed lymphadenectomies.

Immunohistochemical features and clinical-pathological correlations

A complete loss of BAP-1 and PBRM-1 immunostaining was observed in 36.1% (22/61) and 42.6% (26/61) of the cases, respectively, while a totally retained BAP-1 expression (2+ expression) was seen in 37.7% (23/61) of the patients. S100P was negative in 30 out of 61 cases (49.2%), whereas an overexpression (2+ expression) was detected in 19.7% (12/61). miR-31 was absent in just one case, while a mild expression was seen in 60.7% (37/61) and a strong one in 37.7% (23/61) of the patients. The expression status of BAP-1, PBRM-1, S100P, and miR-31 is reported in Table 2.

The statistical analysis showed a strong inverse association between BAP-1 and miR-31 expression ($p = 0.03$). We also found a direct relationship between BAP-1 and S100P staining ($p = 0.02$). S100P expression was significantly higher in the large bile duct subtype of ICC ($p < 0.0001$), thus supporting the adopted histological classification. Moreover, a trend of association was observed between PBRM-1 expression and the presence of perineural invasion ($p = 0.07$). Patients' age, sex, presence or absence of underlying chronic liver diseases, presence or absence of cirrhosis, CEA and CA19-9 levels were not related to the IHC markers expression. There was no association between BAP-1 and PBRM-1, nor between their expression and the grade, the stage or the histological subtype of ICC.

A retained BAP-1 expression (expression = 2+) was associated with a decreased overall (617 days vs 1136 days; $p = 0.04$) and disease-free survival (525 days vs 1373 days; $p = 0.05$), as shown in the Kaplan-Meier curves (Fig. 3a, b). PBRM-1-positive cases demonstrated worse overall (621 days vs 1928 days; $p = 0.002$) and disease-free survivals (491 days vs 1366 days; $p = 0.02$), compared with the PBRM-1-negative ones (Fig. 3c, d). Furthermore, cases with an overexpression of S100P (expression = 2+) showed a significantly reduced overall survival (454 days vs 902 days; $p = 0.005$) (Supplementary Fig. 2).

Interestingly, cases characterized by a retained BAP-1 expression and a concomitant overexpression of S100P showed the worst overall survival (366.5 days), compared to cases with just an overexpression of S100P (519 days), cases with just a retained BAP-1 staining (621 days), and cases with both BAP-1 loss and a negative S100P

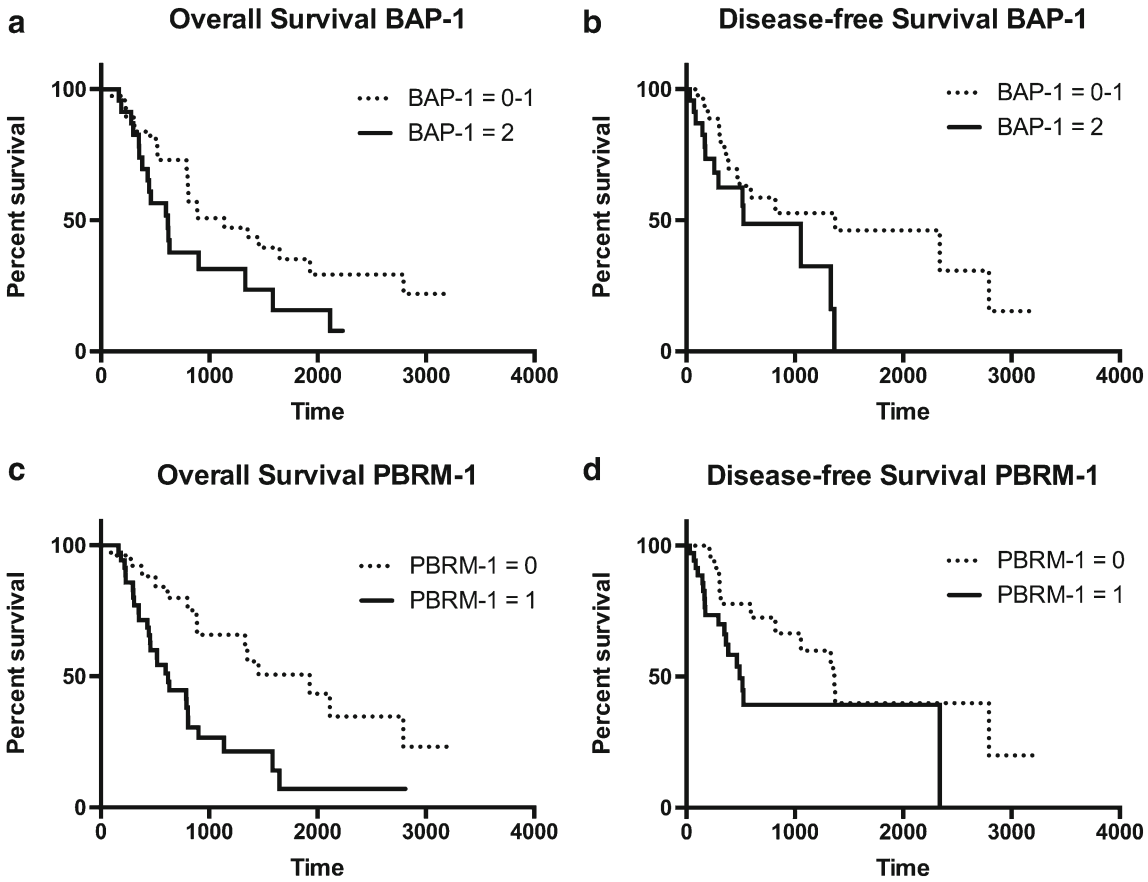

Fig. 3 Kaplan-Meier curves showed a reduced overall and disease-free survival in cases with a retained BAP-1 (**a**, **b**) and PBRM-1 (**c**, **d**) expression

immunostaining (1457 days) ($p = 0.008$). miR-31 expression was not related to patients' prognosis.

The multivariate Cox regression analysis included all the variables that were identified as significant on univariate survival analyses: perineural and vascular neoplastic invasion, T stage, and BAP-1, PBRM-1, and S100P expression. It showed that cases with an overexpression of S100P had a significantly worse overall survival [$p = 0.02$; HR = 1.66 (CI 1.08–2.55)], and that the presence of perineural invasion and a retained PBRM-1 expression are independent predictors of worse overall [$p = 0.02$, HR = 2.25 (CI 1.16–4.39) and $p = 0.001$, HR = 3.13 (CI 1.56–6.28), respectively] and disease-free survivals [$p = 0.03$, HR = 2.43 (CI 1.09–5.4) and $p = 0.03$, HR = 2.51 (CI 1.11–5.67), respectively] (Table 3).

Discussion

Identification of specific ICC prognostic biomarkers is still urgently required [1, 2]. In this study, we evaluated the expression, the clinical-pathological correlations, and the prognostic role of BAP-1, PBRM-1, S100P, and miR-31 in a consecutive mono-Institutional series of ICCs.

For both BAP-1 and PBRM-1, the IHC expression is supposed to be a reliable marker of a double hit inactivation of the gene, which correlates with a loss of nuclear staining [11, 13, 15, 25]. Even if this method has shown very high positive and negative predictive values, a small number of missense mutations may not be detected [11, 15]. In our study, we observed a complete loss of BAP-1 and PBRM-1 expression in 36.1% and 42.6% of cases, respectively. Those frequencies are higher than those previously reported, and, in particular, they are higher than those of *BAP-1* and *PBRM-1* genes mutations identified in previous genomewide analyses [7, 13, 15, 25]. In fact, these studies reported somatic biallelic inactivating mutations (including insertions, deletions, frameshift, nonsense, and missense mutations) within *BAP-1* and *PBRM-1* genes in up to 25% and 17% of ICC, respectively [7, 8, 11, 13, 25]. There could be two possible explanations on these results: i) our interpretation of BAP-1 immunostaining, particularly for those cases with a partial loss of expression (expression = 1+), may not perfectly reflect the mutational status of the gene; ii) there may be a loss of protein expression in the absence of an underlying concurrent genetic mutation. Further studies with molecular analyses are needed to elucidate this matter.

Table 3 Multivariate analysis results

Factors	Overall survival		Disease-free survival	
	HR (95% CI)	*p*	HR (95% CI)	*p*
Perineural invasion	2.25 (1.16–4.39)	0.02	2.43 (1.09–5.4)	0.03
Vascular invasion	1.39 (0.93–2.06)	n. s.	/	/
T stage	0.91 (0.44–1.87)	n. s.	/	/
BAP-1	1.28 (0.87–1.89)	n. s.	1.2 (0.78–1.86)	n. s.
PBRM-1	3.13 (1.56–6.28)	0.001	2.51 (1.11–5.67)	0.03
S100P	1.66 (1.08–2.55)	0.02	/	/

A study from Misumi et al. recently demonstrated that a loss of IHC expression of BAP-1 is related to a better overall and recurrence-free survival in ICC [15]. This interesting association was previously suggested only from another work by Andrici et al. [25], but they just found a strong trend toward improved survival for BAP-1 negative ICCs, while they failed to reach statistical significance. In our study, we observed a significantly decreased overall and disease-free survival in ICCs with a retained BAP-1 expression, compared with those with a loss of staining, confirming that BAP-1 expression is a prognostic factor in ICCs, as previously reported [15].

Overall, these data do not support a tumor suppressor function of *BAP-1* in ICC, suggesting a cell type-specific role for BAP-1, as previously supposed [12]. Of note, several new drugs targeting chromatin remodeling, such as histone deacetylase, DNA methylation and JAK2 inhibitors, that are expected to be effective even in BAP-1 mutated ICCs, deserve further investigation in ICC patients, to determine their effectiveness in spite of the peculiar and different behavior of BAP-1 in this tumor [3, 5, 8, 15, 26].

In their study [15], Misumi et al. reported an association between BAP-1 loss and the small bile duct subtype of ICCs, suggesting that BAP-1 could be a useful marker to determine ICC subtypes. In contrast, in our series, we found a similar prevalence of BAP-1 loss, as well as PBRM-1 loss, in both the ICC subtypes.

miR-31 seems to be involved in the oncogenesis of ICC, and previous studies demonstrated its upregulation in ICC tissue, and its ability to increase cellular proliferation and inhibit cellular apoptosis through several different mechanisms [6, 16, 17]. In our series, we were not able to find any prognostic meaning of miR-31 expression, but we observed an interesting inverse association with BAP-1 expression, supporting what earlier reported in lung cancer [17].

Previous studies also investigated PBRM-1 IHC expression in ICCs, but they were not able to find any significant relationship with clinical-pathological or genetic features [13, 15]. In our study, for the first time, we demonstrated at both uni- and multivariate analyses that a retained PBRM-1 expression is related to a worse outcome, in terms of both overall and disease-free survival, promoting its role as an independent prognostic biomarker in ICC. Furthermore, we find a trend of association between PBRM-1 expression and the presence of perineural invasion. It is known that *PBRM-1* acts mainly as a tumor suppressor gene, regulating many different cell processes, but the whole spectrum of its functions is still poorly understood. Recent studies on renal clear cell carcinoma

specimens suggested new roles for PBRM-1 protein, including the regulation of cytoskeletal reorganization and cell adhesion, and this may help to explain our finding [27].

S100P is a member of the S100 family of calcium-binding proteins and it has been reported to be a diagnostic and prognostic biomarker of ICC, both in liver tissue and bile [6, 18, 19, 21]. Previous IHC studies suggested that it is able to identify that subset of ICCs that probably originates from larger bile ducts, the so called large bile duct subtype, that shares clinical-pathological and molecular features with perihilar and extrahepatic CCs [18, 20–22, 28]. This property of S100P is confirmed in our study, as its expression was strongly increased in large bile duct subtype ICCs. This corroborate the hypothesis that large bile duct subtype ICCs could have the same carcinogenetic pathway of perihilar and extrahepatic CCs, and it suggests that S100P expression could be a helpful tool in differentiating ICC subtypes [20, 21]. As previously reported, in our series, both the uni- and multivariate analyses demonstrated that S100P is an independent predictor of decreased overall survival, further supporting the idea that it could be a useful marker to identify ICC patients with a poor prognosis [20, 21].

In conclusion, our study confirms that a retained BAP-1 expression and an overexpression of S100P are poor prognostic factors, and it demonstrates for the first time that a preserved PBRM-1 expression is related to a worse prognosis in ICC. Therefore BAP-1, PBRM-1, and S100P could be useful biomarkers to identify ICC patients with a poor prognosis. However, as expected for a single-center study, the number of patients in our cohort is not so high, and this can limit the strength of our data. Therefore, larger multicenter studies and a validation in liver biopsy samples are needed to translate these results into the clinical practice. Moreover, the inverse association between BAP-1 and miR-31 expression suggests that miR-31 may act as a direct regulator of BAP-1 even in ICC.

Author contributions Samantha Sarcognato: conception and design, acquisition of data, analysis and interpretation of data, writing and revision of the manuscript;

Enrico Gringeri: conception and design, acquisition of data, analysis and interpretation of data, writing and revision of the manuscript;

Matteo Fassan: conception and design, development of methodology, acquisition of data, analysis and interpretation of data, writing and revision of the manuscript;

Michela Di Giunta: acquisition of data, analysis and interpretation of data;

Valeria Maffeis: analysis and interpretation of data;

Vincenza Guzzardo: development of methodology, technical and material support;

Umberto Cillo: conception and design, interpretation of data, revision of the manuscript;

Maria Guido: conception and design, analysis and interpretation of data, writing and revision of the manuscript, study supervision.

All authors gave final approval for publication.

Maria Guido takes full responsibility for the work as a whole, including the study design, access to data and the decision to submit and publish the manuscript.

Compliance with ethical standards

The study complies with the ethical guidelines of the 1975 Declaration of Helsinki and obtained the approval from the local Ethics Committee (Ethics Committee for Clinical Research—University Hospital of Padova, Italy; protocol #: 0038038/2017).

Informed consent All the patients gave their appropriate informed consent to the surgical procedure.

Conflict of interest The authors declare that they have no conflict of interest.

Ethical responsibilities of authors All of the authors confirm that each of them qualifies for every one of the 4 criteria of authorship.

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