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PBRM1 loss is a late event during the development of cholangiocarcinoma

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Abstract

Somatic mutations in genes encoding chromatin remodelers have been recently reported in several cancer types, including approximately half of cholangiocarcinomas. One of the most commonly mutated chromatin remodelers in cholangiocarcinoma is the *PBRM1* gene located on chromosome 3p21, which encodes a subunit of the SWI/SNF complex. In order to determine the timing of *PBRM1* mutations in biliary carcinogenesis, we used immunohistochemistry to assess PBRM1 protein expression in a series of precursor lesions and invasive biliary carcinomas. Previous studies have correlated loss of protein expression on immunohistochemistry with inactivating mutations in this tumor suppressor gene. We found that PBRM1 loss occurred in approximately 26% of

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CONFLICT OF INTEREST STATEMENT

LDW is a paid consultant for Personal Genome Diagnostics. All the other authors have no potential conflict of interest.

Author contribution: CL, SAR, LDW designed the study; CL, SAR, SMH, RAA, JH, MJW, PA, LDW collected samples; CL, PA, LDW performed morphological analyses; PK performed immunohistochemical staining; CL, PK, LDW evaluated immunohistochemical staining; CL, MF, AP, AN, NV, PC, AS, PK, LDW interpreted the data; CL, LDW wrote manuscript; all coauthors edited and approved the final manuscript.

invasive cancers, but PBRM1 expression was retained in all biliary intraepithelial neoplasia (BilIN) specimens, including 25 intrahepatic BilINs and 19 gallbladder BilINs. These findings indicate that PBRM1 mutation (and resultant loss of expression) is a late event during biliary carcinogenesis. In addition, we also confirm a lack of prognostic significance of PBRM1 status in invasive intrahepatic cholangiocarcinoma. This study provides important insights into the basic mechanisms of chromatin remodeling genes in carcinogenesis.

Keywords

cholangiocarcinoma; BilIN; PBRM1; biliary dysplasia; chromatin remodeling

INTRODUCTION

The genes involved in the establishment of chromatin structure, such as those coding for members of the Switch/Sucrose Non Fermentable (SWI/SNF) complex, are frequently mutated in a number of human cancers [1–7]. A critical member of the SWI/SNF complex is encoded by the *PBRM1* gene (Polybromo-1, pb1) on chromosome 3p21. The PBRM1 protein (also known as BAF180) is a subunit of the SWI/SNF complex and plays an ATP-dependent chromatin-remodeling role, contributing to transcriptional regulation by altering chromatin structure and controlling the accessibility of DNA [8,9].

Somatic mutations in *PBRM1* have been reported in multiple tumor types. Jiao *et al.* first reported that *PBRM1* is frequently mutated in biliary carcinoma, describing somatic mutations of this gene in 17% of intrahepatic cholangiocarcinomas (ICCs) and in 25% of gallbladder carcinomas [2]. An independent study identified *PBRM1* mutations in about 10% of cholangiocarcinomas, with enrichment in ICC [9], while a more recent study investigating mutational status of *PBRM1* with immunohistochemistry reported that 23% of ICCs lack PBRM1 expression [10]. Interestingly, in addition to *PBRM1*, mutations in other genes influencing chromatin organization, including *ARID1A*, *BAP1* and *SMARCB1*, have been identified in cholangiocarcinoma [2,9]. Notably, Jiao *et al.* documented mutations in at least one chromatin-remodeling gene in 47% of cholangiocarcinomas [2], highlighting aberrant chromatin remodeling as a crucial step in biliary carcinogenesis.

Although the presence of somatic *PBRM1* mutations is already well-established in biliary carcinoma, little is known about the timing of *PBRM1* mutations during biliary carcinogenesis. Such knowledge would provide insights into the basic mechanism of tumor suppression by chromatin remodelers in biliary carcinogenesis and may also provide novel strategies for early detection of biliary neoplasia. *PBRM1* mutations are inactivating and result in a loss of protein expression, and previous studies have demonstrated that loss of protein expression on immunohistochemistry strongly correlates with mutational status of *PBRM1*, as well as for other chromatin remodeling genes [10–14]. In order to determine the timing of *PBRM1* mutations, we have assayed PBRM1 protein expression in a series of precursor lesions and invasive carcinomas, including both intrahepatic and gallbladder lesions, representing a broad sample of the spectrum of biliary neoplasia. In addition, since PBRM1 expression has been recently identified as a prognostic factor in several tumor types

[15–17], we have analyzed the correlation of overall survival and PBRM1 status in our ICC cohort.

MATERIALS AND METHODS

Case selection

This study was approved by the Institutional Review Board of The Johns Hopkins Hospital (protocol number NA_00071714, approval date 4/5/2012). All available ICCs diagnosed from 01/01/1994 to 11/01/2014 were retrieved from the Pathology Archives of The Johns Hopkins Hospital. We also retrieved all available cases from liver explants at Johns Hopkins with a diagnosis of hepatolithiasis, primary biliary cirrhosis (PBC), or primary sclerosing cholangitis (PSC) in the period going from 01/01/2004 to 11/01/2014. In order to identify all the possible dysplastic lesions, one pathologist (C.L.) reviewed all the slides of ICC resections and liver explants. A second pathologist (L.D.W.) independently reviewed and blindly evaluated the grade of dysplasia in the selected cases. Finally, all the cases with intrahepatic biliary dysplasia were reviewed at a multi-headed microscope with an expert pathologist (R.H.H.) to obtain a final agreement on the dysplastic lesions.

The biliary dysplasia was classified according to consensus criteria, with three histological grades: biliary intraepithelial neoplasm (BilIN) 1, BilIN-2, and BilIN-3 [18–20]. Similar three-tiered criteria were also employed for classification of mass-forming biliary dysplasia, with distinction of low, intermediate and high grade dysplasia [20].

All cases with a consensus diagnosis of BilIN were analyzed with immunohistochemistry for PBRM1 as described below. In addition, we also included additional ICCs not associated with biliary dysplasia. These cancers were further subclassified in type 1 (also known as large duct variant) and type 2 (also known as small duct variant) ICCs, accordingly to a recently proposed classification based on histological features [21, 22].

Cases of gallbladder dysplasia and carcinoma were obtained from gallbladder resection specimens from The Johns Hopkins Hospital referred to an expert biliary pathologist (P.A.) for classification. These cases were then classified independently by two pathologists (C.L., L.D.W.), using the standardized BilIN classification system [18].

For all the cases with multifocal BilIN, we analyzed only the BilIN with the highest grade of dysplasia.

Immunohistochemistry and survival analysis

Because *PBRM1* mutations result in a loss of protein expression, we used an immunohistochemical approach to study *PBRM1* mutational status [2]. As a further control, we included 6 ICCs with known *PBRM1* mutational status (4 wild type and 2 mutated), which were analyzed in our initial whole exome sequencing study of cholangiocarcinoma [2]. This immunohistochemical method allowed us investigate the mutational status in dysplastic lesions that were too small to be analyzed by sequencing.

Immunohistochemistry for PBRM1 was conducted using the Benchmark XT automated stainer (Ventana), as previously described [11]. Briefly, formalin-fixed, paraffin-embedded tissue sections were cut at 3–4 μ m and air-dried overnight. The sections were deparaffinized, rehydrated, and subjected to heat-induced epitope retrieval with Tris-based cell conditioning buffer solution (Ventana). Sections were then incubated with appropriate primary antibody (Source: Bethyl Laboratories Inc., TX, USA; Type: rabbit polyclonal; Dilution 1:250; Incubation: 32 minutes). For signal detection, ultraView universal detection system (Ventana) was used. The slides were developed using 3–3'-diaminobenzidine chromogen and counterstained with hematoxylin. Appropriate positive and negative controls were utilized for each run of immunostains. Nuclear positivity indicated PBRM1 expression (*PBRM1* wildtype); the lack of staining indicated PBRM1 loss (*PBRM1* mutation). This entire immunohistochemical process for PBRM1 was validated using a cohort of 176 clear cell renal cell carcinomas with known mutation status [2].

The possible prognostic role of PBRM1 expression in our ICC cohort was analyzed with a multivariate analysis adjusted for clinical and pathological features (age, gender, tumor grade, type of surgery, and TNM stage) using a Cox's regression analysis, as well as with a Kaplan-Meier curve.

RESULTS

Case selection

Examination of ICCs lead to identification of 15 associated BilINs (Table 1). Among these, 5 were BilIN-1, 6 were BilIN-2 and 4 were BilIN-3. A similar number of ICCs without associated dysplastic lesions were also included to confirm the mutation prevalence of *PBRM1* in ICC. Although these 14 cases were selected without knowledge of *PBRM1* status, 6 were included in a previous whole exome sequencing study. The analyzed ICCs included 5 type 1 (large duct) carcinomas, 22 type 2 (small duct) carcinomas, and 2 carcinomas with mixed features.

The evaluation of liver explants with hepatolithiasis, PBC, and PSC identified 2 cases of hepatolithiasis with dysplasia (both BilIN-1), 1 case of PBC with dysplasia (BilIN-1), and 6 dysplastic cases among PSC (3 BilIN-1, 2 BilIN-2, and 1 BilIN-3). Representative intrahepatic BilINs are shown in Figure 1 A–C.

The gallbladder cases included 3 cases with invasive adenocarcinoma associated with BilIN (2 BilIN-1 and 1 BilIN-3) (Table 1). Furthermore, 3 cases with a microinvasive adenocarcinoma (<5mm) associated with BilIN were identified: the BilIN were all BilIN-3 [23]. There were also 10 BilIN lesions of the gallbladder not associated with an invasive component: 3 were BilIN-1, 2 were BilIN-2, and 5 were BilIN-3. Representative BilINs from the gallbladder are shown in Figure 1 D–F. Our cohort also included 4 intracystic papillary neoplasms of the gallbladder, 2 with low grade dysplasia and 2 with high grade dysplasia.

Immunohistochemistry and survival analysis

Expression of PBRM1 in neoplastic cells by immunohistochemistry was evaluated with a binary scoring system: lost (immunohistochemistry negative) or retained (immunohistochemistry positive) (Figure 2). Importantly, in our ICC cases with known *PBRM1* mutation status, there was a perfect correlation between immunohistochemical staining and *PBRM1* mutation status: the 2 cases with *PBRM1* mutations showed loss of PBRM1 protein expression, and the 4 wild-type cases retained PBRM1 expression.

The immunohistochemistry results are summarized in Table 2. Among ICC, 7 out of 29 cases (24%) showed loss of PBRM1 on immunohistochemistry (Figure 2); of these, 5 were associated with BilIN and 2 were not. We identified PBRM1 loss in 1 out of 5 type 1 cancers (20%), 5 out of 22 type 2 cancers (23%), and 1 out of 2 mixed type cancers (50%). At the same time, among the 15 biliary dysplasia (BilIN-1,-2 and -3) associated with ICC, no cases presented PBRM1 loss, including the 5 with an associated PBRM1-negative cholangiocarcinoma (Figure 2). The prevalence of PBRM1 loss was significantly higher in cancer compared to BilIN ($p<0.01$, Fisher exact test).

The results of PBRM1 immunohistochemistry in BilINs unassociated with cholangiocarcinoma were similar to those of BilINs associated with cholangiocarcinoma described above. Among the 9 cases of BilIN in hepatolithiasis, PBC, and PSC, no cases showed PBRM1 loss on immunohistochemistry.

Among the gallbladder cases, only 1 case of invasive carcinoma showed PBRM1 loss; the rest of the cases showed a retained expression of PBRM1. Notably, one intracystic papillary neoplasm with high grade dysplasia demonstrated a small portion of the lesion (about 20%) with PBRM1 loss, but in the rest of the lesion PBRM1 was clearly expressed (Figure 3).

Clinical and pathological data for survival analysis was available for 24 ICC patients. With a multivariate analysis, the loss of PBRM1 was not associated with any increased risk of mortality (HR=1.19; 95% CI: 0.06–22.31; $p=0.59$), as also shown in the Kaplan-Meier curve (Figure 4).

DISCUSSION

Our study demonstrates that loss of PBRM1 expression is limited to cases of invasive cancer; neither biliary dysplastic lesions (in the liver or gallbladder) nor microinvasive cancers (in the gallbladder) showed loss of PBRM1 on immunohistochemical analysis. Overall, our results suggest that *PBRM1* mutation is a late event during biliary carcinogenesis in the liver and gallbladder.

ICC can be subclassified into two histological subtypes: large duct type (type 1) and small duct type (type 2) [20, 21, 22]. Recently, Misumi *et al.* reported that *PBRM1* mutations occurred at similar prevalence in both subtypes (42% in type 1 and 53% in type 2), suggesting molecular similarities between the subtypes [10]. Our results are in line with these previous findings, with similar prevalence of PBRM1 loss between the two subtypes. Although the prevalence of PBRM1 loss was lower in our study (20–30% in each group), the

lower number of cases in our series might contribute to this discrepancy. In addition, the results of our survival analysis confirm the finding of Misumi *et al.* that PBRM1 expression does not have a prognostic role in ICC. Notably, in contrast to ICC, PBRM1 expression has been shown to significantly impact survival in other solid malignancies with *PBRM1* mutations [15–17].

It has been hypothesized that biliary carcinoma may develop through two key premalignant precursor lesions: BilIN (flat dysplasia), and intraductal/intracystic papillary neoplasm (mass-forming lesions) [18,19,24,25]. However, somatic mutations in key driver genes have only been identified in BilINs in patients with specific risk factors (i.e. hepatolithiasis) [26]. We hypothesize that this is because the alterations assayed to date are late events in biliary carcinogenesis, but an alternative explanation for the lack of driver alterations is that BilINs are not in fact precursors to cholangiocarcinoma. BilINs have been proposed as a precursor for only type 1 ICC [21,27], but *PBRM1* mutations occur at similar frequency in both subtypes of ICC [10]. The lack of PBRM1 loss in BilINs suggest that alterations in this gene are a late event, at least in the tumorigenesis of type 1 ICC. Of note, there is considerable controversy over the identity of the precursor lesion of type 2 ICC, so it is not possible to address the timing of PBRM1 loss in this subtype. Still, BilINs found in livers with type 2 ICC are relevant – each BilIN is not an obligate precursor of the concomitant carcinoma but instead may be an isolated dysplastic lesion.

Sasaki *et al.* recently reported a study on the expression of another chromatin remodeling gene, *ARID1A*, in cholangiocarcinoma and its precursors [28]. Loss of ARID1A expression was detected by immunohistochemistry biliary carcinomas but not in any of the dysplastic lesions analyzed, which included both BilINs and biliary mass-forming dysplastic lesions [28]. In keeping with our results, this study further highlights that mutations in chromatin remodeling genes are late events in biliary carcinogenesis, occurring predominantly in invasive carcinoma. In contrast, in other organs mutations in chromatin remodelers have been suggested as an early event during carcinogenesis; this has been previously reported for esophageal cancer, as well as ovarian and endometrial cancers [29,30]. This suggests that somatic alterations in chromatin remodelers may play different roles in different organs, as the timing during tumorigenesis is widely disparate.

Notably, in our series, a case of intracystic papillary neoplasm with high-grade dysplasia presented a focal loss of PBRM1 expression (Figure 3); this was the only dysplastic lesion with an abnormal PBRM1 expression. This finding raises the possibility that PBRM1 loss may be involved in earlier stages in the biliary carcinogenesis via mass-forming dysplastic lesions compared to BilIN, and highlights the concept of intra-tumor heterogeneity in pre-neoplastic lesions. Future experiments could assess this heterogeneity with targeted next generation sequencing to confirm the results of the immunohistochemistry. Still, the true prevalence of these early mutations in intracystic papillary neoplasms remains to be determined, as we have only four cases in our cohort.

Our study does have some limitations. Due to the rarity of biliary intraepithelial neoplasia, the number of cases analyzed in this study is limited. This issue is particularly relevant for mass-forming dysplastic lesions, as only four cases of these rare lesions were included in our

study. Our study also excluded intraductal papillary neoplasms of the bile duct, and as such, follow-up studies will be required to determine the role (if any) of PBRM1 loss in these lesions. In addition, our ICC cohort included a relatively small number of type 1 (large duct) ICC, suggesting that the majority of the BilINs analyzed in these patients were concomitant with, rather than precursors to, the co-occurring ICC. Although these cases are still informative, it should be noted that we only analyzed one *PBRM1*-mutant type 1 ICC that had the potential to demonstrate PBRM1 loss in a matched ICC/BilIN pair.

The grading of biliary dysplasia is a subjective process. It is possible that some cases, particularly those diagnosed as low-grade dysplasia (BilIN-1), in fact represent reactive atypia. Such inaccurate categorization could contribute to the low prevalence of PBRM1 alterations demonstrated in our study. However, to minimize the risk of misclassification, we evaluated each case in a rigorous step-wise review involving three pathologists, including experts in biliary neoplasia. Moreover, although it is possible that cases diagnosed as BilIN-1 might truly represent reactive atypia, this is far less likely for higher grades of dysplasia. In our study, all grades of BilIN lacked PBRM1 alterations, suggesting that our conclusions are robust despite the inherent subjectivity in BilIN diagnosis.

In conclusion, based on immunohistochemical analysis of PBRM1 expression in BilIN lesions, we found that *PBRM1* mutations are a late event during cholangiocarcinogenesis. These results have implications for the functional role of chromatin remodeling genes in biliary tumorigenesis.

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Figure 1.

BilINs in liver and gallbladder (H&E images). A. Intrahepatic BilIN-1. B. Intrahepatic BilIN-2. C. Intrahepatic BilIN-3. D. Gallbladder BilIN-1. E. Gallbladder BilIN-2. F. Gallbladder BilIN-3.

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Figure 2. Immunohistochemistry for PBRM1 in dysplastic and malignant biliary lesions. A. Intrahepatic cholangiocarcinoma with retained nuclear PBRM1 expression. B. Intrahepatic cholangiocarcinoma with loss of nuclear PBRM1. This expression pattern is correlated with mutations in the *PBRM1* gene. C. BillIN with retained nuclear PBRM1 expression. No BillIN lesions showed loss of nuclear PBRM1 expression.

Figure 3. Intracystic papillary neoplasm with focal PBRM1 loss. This lesions showed both low-grade dysplasia (A.) and high-grade dysplasia (B.). In the areas of low-grade dysplasia, PBRM1

expression was retained (C.), while expression of PBRM1 was lost in an area with high-grade dysplasia (D.).

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Figure 4. Kaplan-Meier curves showing the survival of ICC patients categorized by PBRM1 expression (dark grey: PBRM1 loss; light grey: PBRM1 retained). The overall survival is not significantly different between these two groups.

Table 1

Biliary dysplasia and carcinoma samples.

Location	Diagnosis	BIIN-1/LGD (# of samples)	BIIN-2/IGD (# of samples)	BIIN-3/HGD (# of samples)	Carcinoma (# of samples)
Intrahepatic	Intrahepatic cholangiocarcinoma	5	6	4	29
	Hepatolithiasis	2	0	0	0
	Primary biliary cirrhosis	1	0	0	0
	Primary sclerosing cholangitis	3	2	1	0
Gallbladder	Invasive adenocarcinoma	2	0	1	3
	Microinvasive adenocarcinoma	0	0	3	3
	Isolated dysplasia	3	2	5	0
	Intracystic papillary neoplasm	2	0	2	0

LGD: low-grade dysplasia; IGD: intermediate-grade dysplasia; HGD: high-grade dysplasia

Table 2

PBRM1 expression in dysplastic and invasive biliary lesions.

Location	Diagnosis	PBRM1 retained # (%)	PBRM1 lost # (%)
Intrahepatic	Carcinoma	22 (76%)	7 (24%)
	BiIN	24 (100%)	0 (0%)
Gallbladder	Carcinoma	5 (83%)	1 (17%)
	BiIN	20 (100%)	0 (0%)