Title

Utility of DNA flow cytometry in distinguishing between malignant and benign intrahepatic biliary lesions

Journal

Virchows Archiv, 477(4)

ISSN

0945-6317

Authors

Wen, Kwun Wah
Rabinovitch, Peter S
Wang, Dongliang
et al.

Publication Date

2020-10-01

DOI

10.1007/s00428-020-02812-w

Peer reviewed

HHS Public Access

Author manuscript

Virchows Arch. Author manuscript; available in PMC 2021 April 01.

Published in final edited form as:

Virchows Arch. 2020 October ; 477(4): 527–534. doi:10.1007/s00428-020-02812-w.

Utility of DNA flow cytometry in distinguishing between malignant and benign intrahepatic biliary lesions

Kwun Wah Wen[1], **Peter S. Rabinovitch**[2], **Dongliang Wang**[3], **Aras N. Mattis**[1], **Linda D. Ferrell**[1], **Won-Tak Choi**[1]

[1]Department of Pathology, University of California at San Francisco, San Francisco, CA 94143, USA

[2]Department of Pathology, University of Washington, Seattle, WA 98195, USA

[3]Department of Public Health and Preventive Medicine, SUNY Upstate Medical University, Syracuse, NY 13210, USA

Abstract

The distinction between well-differentiated intrahepatic cholangiocarcinoma (iCCA) from its morphological mimics such as bile duct adenoma (BDA) and hamartoma (BDH) can be challenging, particularly in small biopsies. Although a few cases of BDA and BDH have been reported to undergo malignant transformation into iCCA, their neoplastic versus benign nature remains debated. DNA flow cytometry was performed on 47 formalin-fixed paraffin-embedded samples of iCCA, 14 BDA, and 18 BDH. Aneuploidy was detected in 22 iCCA (47%) but in none of the 32 BDA and BDH samples. Among the 34 iCCA patients who underwent complete resection and were followed up to tumor recurrence, tumor-related death, or at least for 1 year, the overall recurrence or death rates (regardless of flow cytometric results) were 18, 56, and 71% within 1,3, and 5 years, respectively. The 1-, 3-, and 5-year recurrence or death rates in 18 iCCA patients with aneuploidy were 28, 66, and 66%, respectively, whereas 16 iCCA patients in the setting of normal DNA content had 1-, 3-, and 5-year rates of 6, 44, and 72%, respectively. Although aneuploid tumors were associated with worse outcomes during the first 3 years, this difference was not statistically significant (hazard ratio = 1.4, $p = 0.473$) in the present sample size. In conclusion, the frequency of aneuploidy was significantly higher in iCCA (47%) than in its benign morphological mimics (0%), suggesting that it may potentially serve as a diagnostic marker of malignancy in challenging situations. Our findings also suggest that most BDAs and BDHs, if not all, are benign entities and may not represent precursor lesions to iCCAs that often harbor aneuploidy. Although a larger cohort will be necessary to further determine the prognostic significance of aneuploidy in iCCA patients after resection, the patients with aneuploid tumors may have a higher risk for tumor progression, especially during the first 3 years.

✉Won-Tak Choi, Won-Tak.Choi@ucsf.edu.

Authors' contributions KWW, PSR, ANM, LDF, and WTC contributed to the study concept and design, analysis and interpretation of data, and drafting of the manuscript. WTC and KWW performed the experiments. DW contributed to the analysis and interpretation of data as well as statistical analysis.

Conflict of interest The authors declare that they have no competing interests.

Ethics approval The University of California, San Francisco (UCSF) Institutional Review Board for human subjects research (IRB no. 16–21034) approved our study.

Keywords

Aneuploidy; Bile duct adenoma; Bile duct hamartoma; Cholangiocarcinoma; DNA flow cytometry

Introduction

Intrahepatic cholangiocarcinoma (iCCA) is the second most common primary liver malignancy that often presents at an advanced stage [1–3]. Surgical resection is the mainstay of therapy with curative intent, but even after resection, the prognosis remains poor with 5-year survival rates ranging from 4 to 43% [2]. Well-differentiated iCCA can be challenging to diagnose, particularly in small biopsies, because it may have overlapping histological features with bile duct adenoma (BDA) and hamartoma (BDH). Although largely considered to be benign, BDA and BDH can also demonstrate morphological features concerning for malignancy (including nuclear atypia, stratification of epithelial cells, and irregular glands). In fact, a few cases of BDA [4, 5] and BDH [6, 7] have been reported to undergo malignant transformation into iCCA. Furthermore, a high prevalence of *BRAF* V600E mutations have been reported in BDA (53% versus 0% in BDH), suggesting that BDAs may represent precursors for a subset of iCCAs exhibiting the same mutations [8, 9]. Nonetheless, their neoplastic versus benign nature remains debated.

To aid in the distinction between iCCA and benign intrahepatic biliary lesions, most studies have evaluated immunohistochemical (IHC) stains [10–12]. For instance, an increased Ki-67 proliferation index has been reported in iCCA (mean: 23% versus 1.4% for benign intrahepatic biliary lesions; $p < 0.001$) [12]. Similarly, Tan et al. demonstrated that the expression ofp53, BCL-2, and Ki-67 was greater in iCCA than in benign intrahepatic biliary lesions [11]. However, these studies have shown that the interpretation of staining results is highly variable and subjective (often using a different cutoff for a positive result), and that benign intrahepatic biliary lesions often show weak to moderate positive staining for these IHC markers. Although some molecular markers detected by next-generation sequencing, such as *IDH1/2* mutations (found in 5–36% of iCCA) or *FGFR2-PPHLN1* fusions (5–45%), may be specific for iCCA over other intrahepatic malignancies [13–21], their routine use as a diagnostic marker of malignancy is limited by low sensitivity (5–45%), high cost, and long turnaround time (compared to IHC). Inactivating mutations of BRCA associated protein-1 (BAP1) also occur in iCCA, which can be demonstrated by loss of nuclear staining by IHC. However, loss of BAP1 expression is seen in only 7–25% of iCCA [14, 22].

Alternatively, DNA flow cytometry that measures nuclear DNA content abnormality (i.e., aneuploidy) may potentially serve as an adjunct method in distinguishing between malignant and benign intrahepatic biliary lesions, but such an analysis is not currently available. In this regard, we recently demonstrated the significance of DNA content abnormality as a diagnostic and/or prognostic indicator of malignancy in various settings, including ampullary neoplasia [23], Barrett's esophagus [24], and inflammatory bowel disease [25]. Furthermore, identification of a potential prognostic marker using formalin-fixed paraffin-embedded (FFPE) tumor tissue may be of great value for resected iCCA cases, as it could potentially predict prognosis and guide treatment decisions. As such, in this study, we

evaluated the potential utility of DNA flow cytometry using FFPE tissue in discriminating between iCCA and its morphological mimics, as well as its prognostic potential in predicting outcome of iCCA patients after resection.

Materials and methods

Patients and data collection

After approval by the University of California, San Francisco (UCSF) Institutional Review Board for human subjects research (IRB no. 16–21034), the pathology database (CoPath) was searched from 2000 to 2019 for retrievable cases, which resulted in 47 samples of iCCA from 47 patients (7 needle core biopsies and 40 resections), 14 BDA from 14 patients (1 needle core biopsy and 13 wedge biopsies), and 18 BDH from 17 patients (3 needle core biopsies and 15 wedge biopsies). Perihilar (Klatskin) and distal bile duct carcinoma cases were excluded. All cases were re-reviewed by at least one liver pathologist (WTC, KWW, and LDF) who confirmed the original diagnosis of iCCA, BDA, or BDH. For each case, pertinent clinical data were collected through review of medical records, including age, gender, ethnicity, lesion size (based on the radiological, gross, and/or microscopic measurement), and the presence of primary sclerosing cholangitis (PSC). For iCCA patients, additional histopathological features were recorded, including multifocality, pathological grade, lymphovascular invasion (LVI), perineural invasion (PNI), lymph node (LN) metastasis, and pathological stage.

DNA flow cytometry

As previously described [23–25], 2 to 5 sections of 60-μm thickness were cut from each tissue block, depending on the size of lesion. The sections were manually trimmed to remove as much as uninvolved tissue as possible to enrich a potential abnormal cell population in a background of normal diploid cells. Each sample was stained with DAPI (4,6-diamidino-2-phenylindole; Accurate Chemical & Scientific Corporation, Westbury, NY). The DNA content was measured on a BD LSRII S854 flow cytometer (BD Biosciences, San Jose, CA) with UV laser excitation. All DNA histograms were analyzed using the computer program Multicycle (De Novo software, Glendale, CA) according to the published consensus guidelines [26, 27]. Aneuploidy was defined as an additional G_0/G_1 peak that was visually distinguishable from the normal diploid G_0/G_1 peak. Two authors (WTC and PSR) interpreted all DNA histograms without knowledge of the histological diagnoses.

Statistical analysis

Statistical analysis appropriate for censored data (Kaplan-Meier curves and univariate Cox proportional hazards model) was utilized, since not all patients reached the endpoint of tumor recurrence or tumor-related death before follow-up ended. Only iCCA patients who underwent complete resection and were followed up to recurrence, death, or at least for 1 year were included in this analysis ($n = 34$). The rates of recurrence or death at specific time points were calculated from the Kaplan-Meier curves, and a null hypothesis of equal distribution of detection times was assessed using the log-rank test. Potential demographic and histopathological risk factors (including aneuploidy) were subjected to the univariate

Cox proportional hazards model. Both 95% confidence intervals (CIs) and *p* values were calculated using the asymptotic Wald test. Fisher's exact test was used to compare the rate of aneuploidy in well- to moderately-differentiated versus poorly-differentiated iCCAs, and in small duct versus large duct type iCCAs. A p value < 0.05 was considered to be statistically significant.

Results

DNA content analysis of BDH

The patients consisted of 11 (65%) males and 6 (35%) females with a mean age of 67 years (range 50–83 years) (Table 1). BDHs were usually small with a mean size of 0.4 cm (range 0.2–0.6 cm) and discovered during intra-abdominal surgery for cancer, including esophageal carcinoma (n = 7), pancreatic carcinoma (n = 4), gastric carcinoma (n = 1), colorectal carcinoma (n = 1), and liposarcoma (n = 1). All 18 BDH samples showed normal DNA content (Fig. 1a, b).

DNA content analysis of BDA

Of the 14 patients, 8 (57%) were males and 6 (43%) were females (Table 1). The patients ranged in age from 33 to 68 years, with a mean age of 58 years. The mean size of BDA was 0.5 cm (range 0.2–1 cm). All but 3 cases were biopsied (to rule out metastatic cancer) during surgical resection for esophageal carcinoma (n = 7), pancreatic carcinoma (n = 1), hepatocellular carcinoma (n = 1), gallbladder carcinoma (n = 1), and liposarcoma (n = 1). Similar to BDH, all 14 BDA samples showed no evidence of aneuploidy (Fig. 1c, d).

DNA content analysis of iCCA

The patients included 18 (38%) males and 29 (62%) females with a mean age of 65 years (range 41–82 years) (Table 1). The majority of patients were Caucasians (72%), one of whom had a history of PSC. The mean diameter of iCCA (6.6 cm) was significantly larger than that of BDH (0.4 cm) or BDA (0.5 cm). Thirty-five (74%) of the 47 iCCA cases showed well- to moderately-differentiated tumors, while the remaining 12 (26%) cases were poorly-differentiated. Fourteen (40%) of the 35 well- to moderately-differentiated iCCAs (< 75% solid growth pattern) showed aneuploidy (Fig. 2a, b), whereas 8 (67%) of the 12 poorly-differentiated tumors (≥25% solid growth pattern) demonstrated aneuploidy (p = 0.180). Forty-one (87%) of the 47 iCCA cases showed small- to intermediate-sized tubules and/or anastomosing glands lined by cuboidal to polygonal cells in the peripheral hepatic parenchyma, consistent with small duct type [28, 29]. The remaining 6 iCCAs (13%) showed features of large duct type, as they were found close to the large intrahepatic bile ducts proximal to the hepatic hilum and demonstrated irregularly infiltrating, large, often mucin-secreting glands lined by columnar cells [28, 29]. Twenty-one (51%) of the 41 small duct type iCCAs showed aneuploidy, whereas 1 (17%) of the 6 large duct type iCCAs demonstrated aneuploidy (p = 0.194). Overall, 22 (47%) of the 47 iCCAs showed aneuploidy (Table 1). The mean diameter (7.7 cm) of aneuploid iCCAs (range 3–13.5 cm) was larger than that (5.6 cm) of diploid tumors (range 1.8–14 cm).

A total of 34 iCCA patients underwent complete resection (with negative surgical margin) with at least 1 year follow-up or reached the endpoint of tumor recurrence or tumor-related death after resection. The mean follow-up time was 30 months (range 2–81 months). Among these patients, the overall recurrence or death rates (regardless of flow cytometric results) were 18, 56, and 71% within 1, 3, and 5 years, respectively (95% CIs = [5–31%], [38–73%], and [53–90%], respectively) (Fig. 2c). More interestingly, the 1-, 3-, and 5-year recurrence or death rates in 18 iCCA patients with aneuploidy were 28, 66, and 66%, respectively (95% CIs = [7–49%], [42–90%], and [42–90%], respectively), whereas 16 iCCA patients in the setting of normal DNA content had 1-, 3-, and 5-year rates of 6, 44, and 72%, respectively (Fig. 2d). Twenty-two (65%) of the 34 patients developed recurrent tumor ($n = 15$) or died ($n = 7$) after resection within a mean follow-up time of 22 months (range 2–81 months). Although the 16 patients with normal DNA content had better outcomes during the first 3 years, this prognostic improvement was not statistically significant (hazard ratio [HR] = 1.4, $p = 0.473$) in the present sample size, based on the univariate Cox model with the log-rank test (Table 2). None of the potential demographic risk factors (including age [HR = 1.0, $p = 0.634$], gender [HR = 0.9, $p = 0.820$], ethnicity [HR = 0.5, $p = 0.205$], and the presence of PSC [HR = 0.2, $p = 0.145$]) could significantly predict recurrence-free survival. Similarly, no potential histopathological risk factors, including multifocality (HR = 0.9, $p = 0.944$), tumor size (HR = 1.1, $p = 0.407$), pathological grade (HR = 2.1, $p = 0.302$), LVI (HR = 1.0, $p = 0.947$), PNI ($p = 2.2$, $p = 0.220$), LN metastasis (p = 0.6, $p = 0.365$), and pathological stage (HR = 0.8, $p = 0.711$), demonstrated a significant predictive value. We also performed a statistical analysis using survival or death as the only primary outcome (without including tumor recurrence), but none of the above variables turned out to be a significant prognostic factor for survival (data not shown).

Discussion

The distinction between well-differentiated iCCA and its benign morphological mimics can be challenging, particularly in small biopsies. Well-differentiated iCCA may have overlapping histological features with BDA and BDH, and morphological features suggestive of malignancy (such as irregular glands and stratification of epithelial cells, as well as hyperchromatic and pleomorphic nuclei) can be seen in BDA and BDH. Although IHC stains have been used to facilitate this distinction [10–12], more objective ancillary testing may be useful in better distinguishing iCCA from benign intrahepatic biliary lesions. In this regard, we note that aneuploidy detected by DNA flow cytometry may potentially serve as a diagnostic marker of malignancy, as almost half of iCCA samples (47%) showed aneuploidy (versus 0% in its benign morphological mimics) (Table 1). In support of this, Brunt et al. previously reported that 3 (50%) of 6 iCCA samples showed aneuploid population(s) using DNA image analysis, whereas only diploid populations were present in two BDA samples [30]. Similarly, Kuo et al. demonstrated that approximately 58% of iCCA samples have centrosome abnormalities (which usually result in the formation of multipolar spindles, unbalanced chromosome segregation, and aneuploidy) [31]. Considering that the routine use of molecular alterations seen in iCCA (such as *IDH1/2* mutations or *FGFR2-PPHLN1* fusions) as a diagnostic marker of malignancy is limited by low sensitivity (5–45%), high cost, and long turnaround time (compared to IHC), DNA flow cytometry may

potentially serve as a useful adjunct method in differentiating between iCCA and benign intrahepatic biliary lesions, especially when the initial IHC workup (using Ki-67, p53, and/or BAP1) yields inconclusive results. Also, DNA flow cytometry can be performed on limited biopsy samples (including small needle core and mucosal biopsies) and is a relatively inexpensive, yet objective test that can be completed within 2 days [23–25].

Although no aneuploidy was detected in our cohort of BDAs and BDHs (versus 47% in iCCA) (Table 1), rare cases of BDA [4, 5] and BDH [6, 7] undergoing malignant transformation have been reported in the literature. More recently, Pujals et al. reported that *BRAF* V600E mutations are common in BDA (53%), and that 2 of 4 iCCA cases associated with BDA showed the same mutations [8, 9], suggesting that at least some BDAs are true neoplasms and may represent precursors for a subset of iCCAs exhibiting the same mutations. In this regard, we had one indeterminate case that showed some morphological features of BDA but could not be definitively classified as either benign (BDA or BDH) or malignant (Fig. 3a, b), largely due to the lack of adequate tissue for additional testing. The patient was a 58-year-old man with a 0.2-cm subcapsular liver lesion identified during the surgical resection of gastric signet-ring cell carcinoma (Fig. 3c). Although the overall (well-circumscribed) shape of the lesion, peripheral inflammatory changes, looser stroma, and lack of any visible portal tracts could be somewhat more suggestive of some variants of BDA than BDH [9, 32, 33], the focal luminal dilatations with possible bile could also suggest a BDH [9, 32] and these same features noted above, plus the degree of nuclear atypia present, could also fall within the spectrum of a possible well-differentiated adenocarcinoma (such as iCCA or metastatic adenocarcinoma from a different primary site), or perhaps a lesion that is transitioning to iCCA. Interestingly, this lesion showed a distinct aneuploid population concerning for malignancy (Fig. 3d), but there was no significantly elevated Ki-67 proliferative index (< 1%, Fig. 3e) or BRAF V600E expression (Fig. 3f) to further support the diagnosis of malignancy or BDA [9, 12]. Additional molecular (i.e., *BAP1* and *IDH1/2* mutations, or *FGFR2-PPHLN1* fusions found in iCCA) and/or IHC analyses could have been helpful in this setting, but the lack of adequate tissue precluded its definitive diagnosis. Overall, we could not entirely exclude the possibility that this case may represent an early, small “incipient” iCCA, an (not yet defined) in situ iCCA, or transitional process from dysplasia to carcinoma. Given the low Ki-67, and the poorly-differentiated nature of the gastric primary adenocarcinoma in this case, we do not favor this lesion as an extremely well-differentiated metastatic adenocarcinoma from the gastric primary. However, a clinically “benign” intrahepatic biliary lesion not diagnostically malignant, but instead described as an atypical ductular lesion rather than a variant of BDA or BDH, is still a possibility. Additional larger studies may be helpful to better define early, small “incipient” iCCAs and to determine if aneuploidy can occur in BDA and/or BDH. For now, if aneuploidy is detected in an intrahepatic glandular/biliary lesion, careful follow-up (or possibly complete excision) may be warranted to prevent potential risk for concurrent or subsequent iCCA or metastatic adenocarcinoma.

In the present study, although patients with diploid iCCAs showed a better prognosis than those with aneuploid tumors during the first 3 years, this prognostic improvement did not reach statistical significance (HR = 1.4, $p = 0.473$) (Table 2 and Fig. 2d). However, there is strong evidence that DNA index is an important prognostic tool for iCCA patients. For

instance, in a prospective study of 65 iCCA patients undergoing resection, Kamphues et al. demonstrated that diploid tumors ($p = 0.024$) and lower tumor stage ($p = 0.017$) were significantly associated with improved survival, whereas all other histopathological factors had no prognostic value [34]. Similarly, Abou-Rebyeh et al. reported that DNA ploidy is the most accurate prognostic factor for survival of patients with hilar cholangiocarcinoma after resection [35]. In that study, 75% of patients with diploid tumors survived more than 3 years, whereas all patients with aneuploid tumors died within 18 months. This is in agreement with our finding that 56% of our patients with diploid tumors survived without recurrence within the first 3 years (versus 34% of patients with aneuploid tumors) (Fig. 2d). The lack of statistical significance in our cohort is most likely due to the aggressive nature of these tumors at the time of presentation and/or the limited sample size ($n = 34$). In fact, even after complete resection, 65% of our patients developed recurrent tumor ($n = 15$) or died ($n = 7$) within a mean follow-up time of 22 months. This is consistent with the previous finding that even after resection, recurrence was observed in 62% of iCCA patients within a median follow-up of 26 months [1]. Nevertheless, if these earlier and our results are confirmed by larger studies, an aggressive surgical management may be warranted in patients with diploid tumors, because resection offers very good chance for long-term survival for these patients, whereas patients with aneuploid tumors may need additional neoadjuvant therapy in addition to resection.

In conclusion, our results demonstrate the promise of DNA flow cytometry using FFPE tissue in distinguishing between malignant and benign intrahepatic biliary lesions, as 47% of iCCA samples showed aneuploidy (versus 0% in BDA and BDH). Our findings also suggest that most BDAs and BDHs, if not all, are benign entities and may not represent precursor lesions to iCCAs that often harbor aneuploidy. However, given the one atypical lesion that was identified not diagnostic for benign or malignant process, aneuploidy may prove to be an early marker for a transitional/dysplastic, or incipient low grade adenocarcinoma in this setting. Although a larger cohort will be necessary to further determine the prognostic significance of aneuploidy in iCCA patients, the presence of aneuploidy may identify a subgroup of patients who are at higher risk for tumor progression, especially during the first 3 years.

Funding

This study was funded by the UCSF Department of Pathology and further supported by funds awarded to the UCSF Liver Center (NIH P30 DK026743).

References

1. Endo I, Gonen M, Yopp AC, Dalal KM, Zhou Q, Klimstra D, D'Angelica M, DeMatteo R, Fong Y, Schwartz L, Kemeny N, O'Reilly E, Abou-Alfa GK, Shimada H, Blumgart LH, Jarnagin WR (2008) Intrahepatic cholangiocarcinoma: rising frequency, improved survival, and determinants of outcome after resection. Ann Surg 248:84–96 [PubMed: 18580211]
2. Malhi H, Gores GJ (2006) Review article: the modern diagnosis and therapy of cholangiocarcinoma. Aliment Pharmacol Ther 23: 1287–1296 [PubMed: 16629933]
3. Esnaola NF, Meyer JE, Karachristos A, Maranki JL, Camp ER, Denlinger CS (2016) Evaluation and management of intrahepatic and extrahepatic cholangiocarcinoma. Cancer 122:1349–1369 [PubMed: 26799932]

4. Hasebe T, Sakamoto M, Mukai K et al. (1995) Cholangiocarcinoma arising in bile duct adenoma with focal area of bile duct hamartoma. Virchows Arch 462:209–213
5. Pinho AC, Melo RB, Oliveira M, Almeida M, Lopes J, Graça L, Costa-Maia J (2012) Adenoma-carcinoma sequence in intrahepatic cholangiocarcinoma. Int J Surg Case Rep 3:131–133 [PubMed: 22326450]
6. Jain D, Sarode VR, Abdul-Karim FW, Homer R, Robert ME (2000) Evidence for the neoplastic transformation of Von-Meyenburg complexes. Am J Surg Pathol 24:1131–1139 [PubMed: 10935654]
7. Song JS, Lee YJ, Kim KW, Huh J, Jang SJ, Yu E (2008) Cholangiocarcinoma arising in von Meyenburg complexes: report of four cases. Pathol Int 58:503–512 [PubMed: 18705771]
8. Pujals A, Amaddeo G, Castain C, Bioulac-Sage P, Compagnon P, Zucman-Rossi J, Azoulay D, Leroy K, Zafrani ES, Calderaro J (2015) BRAF V600E mutations in bile duct adenomas. Hepatology 61:403–405 [PubMed: 24634053]
9. Pujals A, Bioulac-Sage P, Castain C, Charpy C, Zafrani ES, Calderaro J (2015) BRAF V600E mutational status in bile duct adenomas and hamartomas. Histopathology 67:562–567 [PubMed: 25704541]
10. Bertram S, Padden J, Kälsch J, Ahrens M, Pott L, Canbay A, Weber F, Fingas C, Hoffmann AC, Vietor A, Schlaak JF, Eisenacher M, Reis H, Sitek B, Baba HA (2016) Novel immunohistochemical markers differentiate intrahepatic cholangiocarcinoma from benign bile duct lesions. J Clin Pathol 69:619–626 [PubMed: 26729014]
11. Tan G, Yilmaz A, De Young BR et al. (2004) Immunohistochemical analysis of biliary tract lesions. Appl Immunohistochem Mol Morphol 12:193–197 [PubMed: 15551730]
12. Tsokos CG, Krings G, Yilmaz F, Ferrell LD, Gill RM (2016) Proliferative index facilitates distinction between benign biliary lesions and intrahepatic cholangiocarcinoma. Hum Pathol 57:61–67 [PubMed: 27396933]
13. Sia D, Losic B, Moeini A et al. (2015) Massive parallel sequencing uncovers actionable FGFR2-PPHLN1 fusion and ARAF mutations in intrahepatic cholangiocarcinoma. Nat Commun 6:6087 [PubMed: 25608663]
14. Jiao Y, Pawlik TM, Anders RA, Selaru FM, Streppel MM, Lucas DJ, Niknafs N, Guthrie VB, Maitra A, Argani P, Offerhaus GJA, Roa JC, Roberts LR, Gores GJ, Popescu I, Alexandrescu ST, Dima S, Fassan M, Simbolo M, Mafficini A, Capelli P, Lawlor RT, Ruzzenente A, Guglielmi A, Tortora G, de Braud F, Scarpa A, Jarnagin W, Klimstra D, Karchin R, Velculescu VE, Hruban RH, Vogelstein B, Kinzler KW, Papadopoulos N, Wood LD (2013) Exome sequencing identifies frequent inactivating mutations in BAP1, ARID1A and PBRM1 in intrahepatic cholangiocarcinomas. Nat Genet 45:1470–1473 [PubMed: 24185509]
15. Kendall T, Verheij J, Gaudio E et al. (2019) Anatomical, histomorphological and molecular classification of cholangiocarcinoma. Liver Int 39:7–18 [PubMed: 30882996]
16. Nakamura H, Arai Y, Totoki Y, Shirota T, Elzawahry A, Kato M, Hama N, Hosoda F, Urushidate T, Ohashi S, Hiraoka N, Ojima H, Shimada K, Okusaka T, Kosuge T, Miyagawa S, Shibata T (2015) Genomic spectra of biliary tract cancer. Nat Genet 47:1003–1010 [PubMed: 26258846]
17. Borger DR, Tanabe KK, Fan KC, Lopez HU, Fantin VR, Straley KS, Schenkein DP, Hezel AF, Ancukiewicz M, Liebman HM, Kwak EL, Clark JW, Ryan DP, Deshpande V, Dias-Santagata D, Ellisen LW, Zhu AX, Iafrate AJ (2012) Frequent mutation of isocitrate dehydrogenase (IDH)1 and IDH2 in cholangiocarcinoma identified through broad-based tumor genotyping. Oncologist 17:72–79 [PubMed: 22180306]
18. Liau JY, Tsai JH, Yuan RH et al. (2014) Morphological subclassification of intrahepatic cholangiocarcinoma: etiological, clinicopathological, and molecular features. Mod Pathol 27:1163–1173 [PubMed: 24406866]
19. Macias RIR, Kornek M, Rodrigues PM et al. (2019) Diagnostic and prognostic biomarkers in cholangiocarcinoma. Liver Int 39:108–122 [PubMed: 30843325]
20. Arai Y, Totoki Y, Hosoda F, Shirota T, Hama N, Nakamura H, Ojima H, Furuta K, Shimada K, Okusaka T, Kosuge T, Shibata T (2014) Fibroblast growth factor receptor 2 tyrosine kinase fusions define a unique molecular subtype of cholangiocarcinoma. Hepatology 59:1427–1434 [PubMed: 24122810]

21. Wang P, Dong Q, Zhang C, Kuan PF, Liu Y, Jeck WR, Andersen JB, Jiang W, Savich GL, Tan TX, Auman JT, Hoskins JM, Misher AD, Moser CD, Yourstone SM, Kim JW, Cibulskis K, Getz G, Hunt HV, Thorgeirsson SS, Roberts LR, Ye D, Guan KL, Xiong Y, Qin LX, Chiang DY (2013) Mutations in isocitrate dehydrogenase 1 and 2 occur frequently in intrahepatic cholangiocarcinomas and share hypermethylation targets with glioblastomas. Oncogene 32:3091–3100 [PubMed: 22824796]
22. Simbolo M, Fassan M, Ruzzenente A et al. (2014) Multigene mutational profiling of cholangiocarcinomas identifies actionable molecular subgroups. Oncotarget 5:2839–2852 [PubMed: 24867389]
23. Wen KW, Kim GE, Rabinovitch PS, Wang D, Mattis AN, Choi WT (2019) Diagnosis, risk stratification, and management of ampullary dysplasia by DNA flow cytometric analysis of paraffin-embedded tissue. Mod Pathol 32:1291–1302 [PubMed: 30976103]
24. Choi WT, Tsai JH, Rabinovitch PS, Small T, Huang D, Mattis AN, Kakar S (2018) Diagnosis and risk stratification of Barrett's dysplasia by flow cytometric DNA analysis of paraffin-embedded tissue. Gut 67:1229–1238 [PubMed: 28642331]
25. Tsai JH, Rabinovitch PS, Huang D et al. (2017) Association of aneuploidy and flat dysplasia with development ofhigh-grade dysplasia or colorectal cancer in patients with inflammatory bowel disease. Gastroenterology 153:1492–1495 [PubMed: 28843957]
26. Rabinovitch P (1992) Practical considerations for DNA content and cell cycle analysis. In: Bauer KD, Duque RE, Shankey TV (eds) Clinical flow cytometry: principles and applications. Williams and Wilkins, Baltimore, pp 117–142
27. Shankey TV, Rabinovitch PS, Bagwell B, Bauer KD, Duque RE, Hedley DW, Mayall BH, Wheeless L, Cox C (1993) Guidelines for implementation of clinical DNA cytometry. International Society for Analytical Cytology. Cytometry 14:472–477 [PubMed: 8354117]
28. Nakanuma Y, Klimstra DS, Komuta M et al. (2018) Intrahepatic cholangiocarcinoma. In: The WHO Classification of Tumours Editorial Board (ed) WHO Classification of Tumours Digestive System Tumours. International Agency for Research on Cancer (IARC), Lyon, pp 254–259
29. Sigel CS, Drill E, Zhou Y, Basturk O, Askan G, Pak LM, Vakiani E, Wang T, Boerner T, Do RKG, Simpson AL, Jarnagin W, Klimstra DS (2018) Intrahepatic cholangiocarcinomas have histologically and immunophenotypically distinct small and large duct patterns. Am J Surg Pathol 42:1334–1345 [PubMed: 30001234]
30. Brunt EM, Kraemer BB (1996) DNA image analysis study of lesions of the gallbladder and biliary system. Liver Transpl Surg 2: 284–289 [PubMed: 9346662]
31. Kuo KK, Sato N, Mizumoto K, Maehara N, Yonemasu H, Ker CG, Sheen PC, Tanaka M (2000) Centrosome abnormalities in human carcinomas of the gallbladder and intrahepatic and extrahepatic bile ducts. Hepatology 31:59–64 [PubMed: 10613729]
32. Tsui WM, Nakanuma Y (2018) Bile duct adenoma. In: The WHO Classification of Tumours Editorial Board (ed) WHO Classification of Tumours Digestive System Tumours. International Agency for Research on Cancer (IARC), Lyon, pp 245–247
33. Allaire GS, Rabin L, Ishak KG, Sesterhenn IA (1988) Bile duct adenoma. A study of 152 cases. Am J Surg Pathol 12:708–715 [PubMed: 3046396]
34. Kamphues C, Al-Abadi N, Dürr A et al. (2014) DNA index is a strong predictive marker in intrahepatic cholangiocarcinoma: the results of a five-year prospective study. Surg Today 44:1336–1342 [PubMed: 23975588]
35. Abou-Rebyeh H, Al-Abadi H, Jonas S et al. (2002) DNA analysis of cholangiocarcinoma cells: prognostic and clinical importance. Cancer Detect Prev 26:313–319 [PubMed: 12430636]

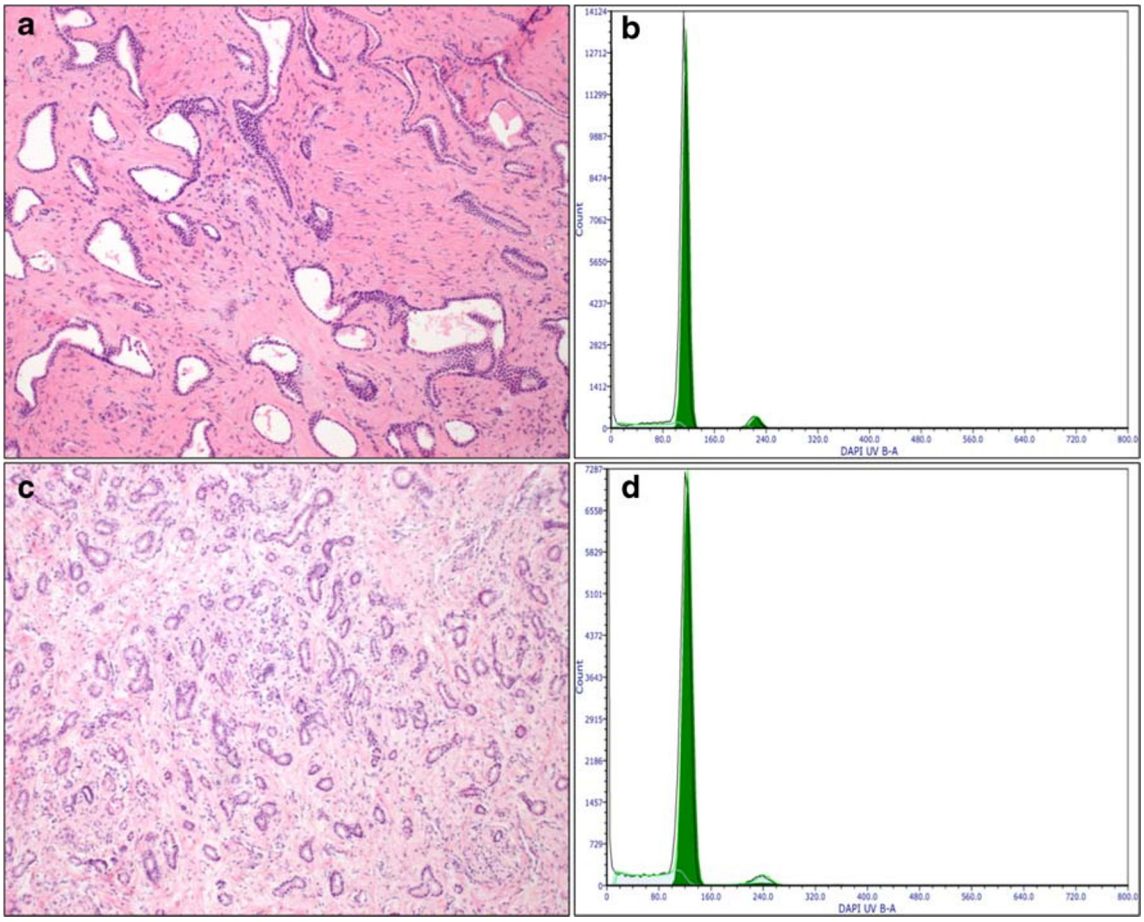

Fig. 1.

a, b Bile duct hamartoma consists of irregularly dilated bile ducts embedded in a dense fibrous stroma (hematoxylin and eosin [H & E], ×10). Intraluminal bile juice is noted in some lumens. The DNA histogram shows a normal diploid population (green). (**c, d**) Bile duct adenoma is composed of closely packed, small, uniformly sized bile ducts lined by single cuboidal cells interspersed in a fibrous stroma (H & E, ×10). No significant nuclear atypia or mitotic figures are present. A normal diploid population (green) is present in the DNA histogram

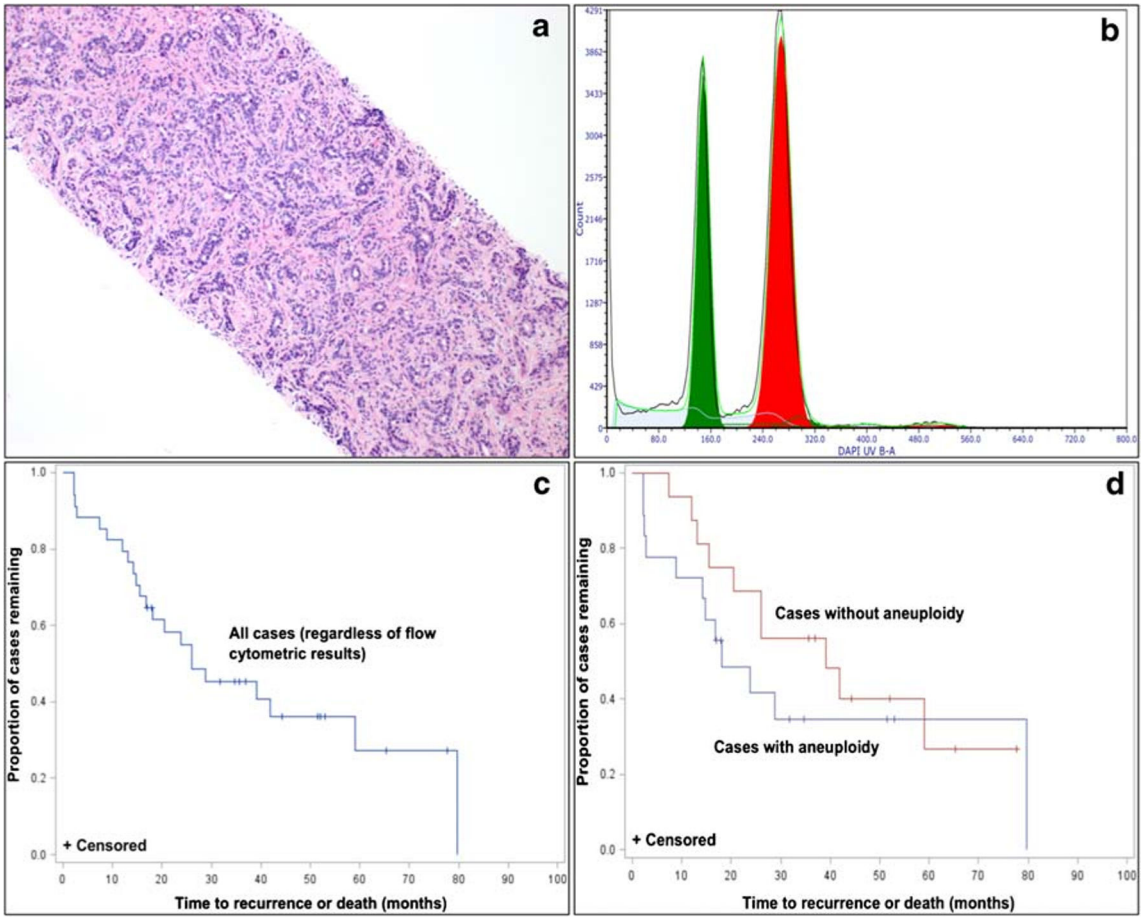

Fig. 2.

a, b Well-differentiated intrahepatic cholangiocarcinoma (iCCA) shows overlapping histological features with BDA (H & E, ×10). Tumor cells are lined by a single layer of cuboidal cells with mild nuclear atypia. No obvious desmoplastic stroma, intracytoplasmic mucin, or perineural or lymphovascular invasion is identified. The DNA histogram shows a distinct aneuploid population (red) in addition to the normal diploid peak (green). **c** Among 34 iCCA patients who underwent complete resection and were followed up to tumor recurrence, tumor-related death, or at least for 1 year, the overall recurrence or death rates (regardless of flow cytometric results) were 18, 56, and 71% within 1,3, and 5 years, respectively **d** While 16 iCCA patients in the setting of normal DNA content had 1-, 3-, and 5-year recurrence or death rates of 6, 44, and 72%, respectively, the 1-, 3-, and 5-year rates in 18 iCCA patients with aneuploidy were 28, 66, and 66%, respectively

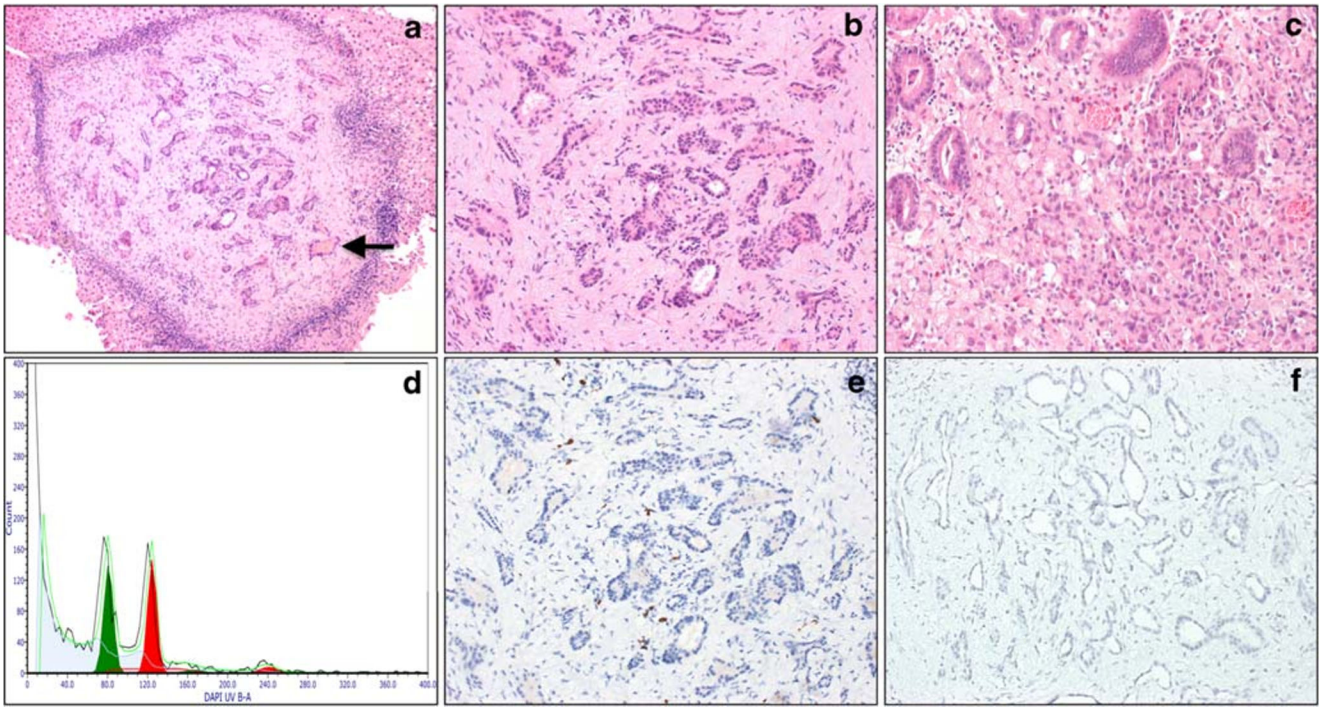

Fig. 3.

a, b The relatively well-circumscribed nature of this lesion, an inflammatory component at the junction between the lesion and adjacent normal liver parenchyma, and looser stroma could be suggestive of some variants of BDA (**a**, H & E, ×10), but the presence of irregular glands with some nuclear atypia as well as focal luminal dilatations with possible bile (arrow) raised consideration for BDH or possibly well-differentiated adenocarcinoma (such as iCCA or metastatic adenocarcinoma from a different primary site) (**b**, H & E, ×20). **c** The patient's primary tumor, gastric signet-ring cell carcinoma, is morphologically distinct from the liver lesion (H & E, ×20). **d-f** The DNA histogram of the liver lesion shows a distinct aneuploid population (red) concerning for malignancy (**d**), but no significantly elevated Ki-67 proliferative index (< 1%, **e**) or BRAF V600E expression (**f**) was observed

Table 1

Patient demographics and lesion characteristics

	Overall (*n* = 79, 78 patients)	Bile duct hamartoma (*n* = 18, 17 patients)	Bile duct adenoma (*n* = 14, 14 patients)	Cholangiocarcinoma (*n* = 47, 47 patients)
Mean age, years (range)	64 (33–83)	67 (50–83)	58 (33–68)	65 (41–82)
Male (%)	37 (47%)	11 (65%)	8 (57%)	18 (38%)
Caucasian (%)	63 (81%)	16 (94%)	13 (93%)	34 (72%)
Size, cm (range)	4.2 (0.2–14)	0.4 (0.2–0.6)	0.5 (0.2–1)	6.6 (1.8–14)
Primary sclerosing cholangitis (%)	1 (1%)	0 (0%)	0 (0%)	1 (2%)
Aneuploidy (%)	22 (28%)	0 (0%)	0 (0%)	22 (47%)

Table 2

Univariate Cox proportional hazards model with tumor recurrence or tumor-related death as the outcome in patients with intrahepatic cholangiocarcinoma (n = 34)

Outcome of cholangiocarcinoma			
	Recurrence or death		
Univariate Cox model	***p* value**	**Hazard ratio**	**95% confidence interval**
Aneuploidy	0.473	1.4	0.6–3.3
Age	0.634	1.0	0.9–1.0
Gender	0.820	0.9	0.4–2.2
Ethnicity	0.205	0.5	0.2–1.4
Primary sclerosing cholangitis	0.145	0.2	0.0–1.7
Multifocality	0.944	0.9	0.2–4.2
Size	0.407	1.1	0.9–1.2
Pathological grade	0.302	2.1	0.5–8.3
Lymphovascular invasion	0.947	1.0	0.4–2.4
Perineural invasion	0.220	2.2	0.6–7.3
Lymph node metastasis	0.365	0.6	0.2–1.8
Pathological stage	0.711	0.8	0.2–3.4