Testing for ROS1, ALK, MET, and HER2 rearrangements and amplifications in a large series of biliary tract adenocarcinomas

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Acknowledgements

This work was financially supported by AFEF (Association Française pour l'Etude du Foie).

We thank Sylvie Dumont and Fatiha Merabtene ("Plateforme d'Histomorphologie St-Antoine", Sorbonne Université, UMS30 LUMIC) for technical assistance.

Abstract

Biliary tract carcinomas are divided into intrahepatic, perihilar, distal extrahepatic cholangiocarcinomas, and gallbladder adenocarcinomas. Therapies targeting *ROS1*, *ALK*, *MET* and *HER2* alterations are currently evaluated in clinical trials. We assessed *ROS1*, *ALK* translocations/amplifications as well as *MET*, and *HER2* amplifications for each tumor subtype by fluorescent in situ hybridization (FISH) and immunohistochemistry (IHC) in 73 intrahepatic, 40 perihilar bile duct , 36 distal extrahepatic cholangiocarcinomas, and 45 gallbladder adenocarcinomas (n=194). By FISH, we detected targetable alterations in 5.2% of cases (n=10): *HER2* and *MET* amplifications were found in 4.1% (n=8) and 1.0% (n=2), respectively. The *HER2* amplified cases were mostly gallbladder adenocarcinomas (n = 5,). The *MET* and *HER2* amplified cases were all positive by IHC. Fourteen cases without *MET* amplification were positive by IHC, whereas HER2 over-expression was detected by IHC only in *HER2* amplified cases. We detected no *ALK* or *ROS1* translocation or amplification. Several alterations were consistent with aneuploidy: 24 cases showed only one copy of *ROS1* gene, 4 cases displayed a profile of chromosomal instability, and an over-representation of centromeric alpha-satellite sequences was found in five cases.

We confirm a relatively high rate of *HER2* amplifications in gallbladder adenocarcinomas and the efficacy of IHC to screen these cases. Our results also suggest the value of IHC to screen *MET* amplification. Contrary to initial publications, *ROS1* rearrangements seem to be very rare in biliary tract adenocarcinomas. We confirm a relatively high frequency of aneuploidy and chromosomal instability, and reveal the over-representation of centromeric alpha-satellite sequences in intrahepatic cholangiocarcinomas. .

Keywords:

Biliary tract adenocarcinoma; *HER2; MET; ROS1*; fluorescent in situ hybridization; immunohistochemistry.

Introduction

Although biliary tract adenocarcinoma is a rare cancer (five cases per 100,000 inhabitants in industrialized countries) [1], its incidence has been increasing during the last thirty years particularly for intrahepatic cholangiocarcinomas [2]. Biliary tract adenocarcinomas can rarely be surgically resected and more than 50% of the patients will relapse [3]. The use of adjuvant treatments is still controversial with a median survival of only 9 to 15 months given the chemoresistance of this cancer [4]. In this context, other therapies are needed and characterization of molecular alterations that may be potential targets for therapy is crucial.

Several theranostic alterations have been targeted in clinical trials like ACSé (NCT02034981) evaluating crizotinib safety and efficacy in patients with locally advanced rearranged cancers with *ALK*, *MET*, and *ROS1* rearrangements, or NCT02836847 and NCT03093870 trials that investigated trastuzumab and varlitinib efficacy in HER2 amplified biliary tract adenocarcinomas [5].

However, there are few data regarding these alterations in biliary tract adenocarcinomas and previous studies reported contradictory results (Table 1 of the supplementary data) [6-19]. FIG-ROS1 fusions were detected by PCR in 8.7% cholangiocarcinomas [6] and 9.2%, biliary tract carcinomas (exclusively in extrahepatic biliary cholangiocarcinomas and gallbladder carcinomas)[7]. The frequency of ROS1 rearrangements was lower with 3 cases of *ROS1* rearrangements out of 261 cholangiocarcinomas (1.1%) using fluorescent in situ hybridization (FISH), and exclusively in intrahepatic tumors [8]. A recent report mentioned the absence of *ROS1* rearrangement out of 110 biliary tract adenocarcinomas assessed by FISH [9]. In this study, one case with *ALK* rearrangement was detected using FISH and *MET* expression was explored using only immunohistochemistry (IHC). *HER2* amplification has been described in biliary tract adenocarcinomas with a frequency around 3% in distal extrahepatic cholangiocarcinomas and 10% in gallbladder carcinomas [10, 12, 13, 16, 18-21].

Biliary tract adenocarcinomas are gathered together in studies although it is well known that they do not represent a single tumor type, as they arise from different biliary cells and display different mutational profiles [5, 22-24]. They are classified according to their anatomical location into intrahepatic, extrahepatic cholangiocarcinomas (perihilar or distal) and gallbladder carcinomas. Morphomolecular subtypes (small and large duct type) are now integrated for intrahepatic cholangiocarcinomas in the fifth edition of the WHO classification of digestive system tumors, [25, 26]. Therefore, in order to have a precise view of some molecular targets in biliary tract adenocarcinomas, we assessed *ROS1* and *ALK* rearrangements and amplifications, as well as *MET* and *HER2* amplifications using FISH as gold standard in a large French series of biliary tract carcinomas that are well-

characterized in terms of location, pathology and staging. We also performed immunohistochemical stainings in order to determine which method, IHC or FISH, was more effective for screening these tumors.

Patients and methods

Case selection

One hundred and ninety-four (194) cases of histologically-confirmed biliary tract adenocarcinomas diagnosed on surgically-resected specimens between 2005 and 2019 were retrieved from the Saint-Antoine Hospital database. Only cases with invasive carcinomas were included. Informed consent was obtained for the study.

Age, gender, tumor location, associated risk factors of liver or biliary tract disease were recorded. Tumor stage, nodal status, vascular invasion, perineural invasion and status of the resection margins were retrieved from pathologic reports. Tumor stage was defined for all cases according the 7th edition of the TNM classification.

Biliary tract adenocarcinoma specimens and tissue microarray construction

All specimens were formalin-fixed and paraffin-embedded. Based on radiologic and pathologic findings, biliary tract adenocarcinomas were subdivided into four subtypes according to their location: intrahepatic, perihilar bile duct, distal extrahepatic bile duct cholangiocarcinomas, and gallbladder adenocarcinomas. For each case, one representative tumor block was selected for tissue microarray construction. Two to five 0.6-mm tissue cores were used and hematoxylin and eosin (HE) staining was performed on each tissue microarray slide to confirm the presence of tumor areas. Whole tissue sections were used for the 36 distal extrahepatic bile duct cholangiocarcinomas.

Morphological analysis of the tumors

HE-stained sections were reviewed to specify histological type and grade according to the WHO classification [27]. We also subclassified intrahepatic cholangiocarcinomas into cholangiolar and bile duct type using morphological criteria described in Liau et al. [25], which are similar to the WHO criteria's defining the small and large duct types [26].

Fluorescent in situ hybridization for ROS1, ALK, MET and HER2,

Probes and technical details for each probe are listed in Table 2 of the supplementary data. FISH was performed on tissue microarrays using six probes to detect *ROS1* rearrangement or amplification, *cMET* amplification, *HER2*

amplification, *ALK* rearrangement or amplification, according to the manufacturers' instructions. Briefly, whatever the probe used, 3-µm slides from tissue microarray blocks were deparaffinized using xylene washes. After rehydration, slides were microwaved preprocessed and after pepsine digestion, dried before co-denaturation and overnight hybridization using a hybridizer system (Dako, Agilent, CA, United States). Post-hybridization washes and counterstain were performed according to each of the probes that were used.

For *ROS1* break-apart probes detection, FISH slides were scanned using PathScan® FISH (Excilone, EXCS-PS-F, Elancourt, France) at x60 objective, after delimitating regions of interest on HE slides. Scanned FISH slides were visualized on PathScan® Viewer (Excilone, Elancourt, France), selecting appropriate channels. FISH signal abnormalities were confirmed using a fluorescence microscope with appropriate channels. For all other probes, FISH slides were directly visualized on fluorescence microscope. Forty non-overlapping nuclei from three tumor areas were evaluated for each probe at x100 oil immersion objective. *ROS1* translocation was defined as the presence of green and red signals separated by at least two signal diameters in the cell nucleus, with still a fused red and green signal. *ROS1* monosomy was defined as a unique fused red and green signal with two *HER2*, *MET* and *ALK* signals, in most nuclei. *HER2* amplification was defined by *HER2*/CEP17 ratio higher than 2, with no *HER2* signals less than 6 [28]. As proposed by some authors for lung adenocarcinoma, *MET* amplification was defined by the existence of 6 copies or more of *MET* per cell [29], and because the probe used did not target centromere 7, *MET*/CEP7 ratio was not evaluated. Because there are no official guidelines to evaluate *ROS1* amplification, we defined *ROS1* amplification as for *MET*. *ALK* amplification was defined by an *ALK*/CEP2 ratio higher than 2 [30].

Aneuploidy was defined by an unbalanced number of chromosomes or loss of large portions of chromosomes; polysomy as a type of aneuploidy, with three or more copies of one or more chromosomes. Because we used dual probes, one locus-specific and one centromeric, one fused signal loss might suggest whole or segmental chromosomal aneuploidy or monosomy [31].

Unbalanced ratios of specific gene loci and centromeric alpha-satellite sequences were analyzed, by enumeration of the fluorescent signals in the tumor cells, considering cell-to-cell variability, a feature of dynamic chromosomal instability [32].

Centromeric alpha-satellite sequences over-representation was defined as a number of alpha-satellites signals greater than 2 with a number of specific genes loci signals equal to 2.

IHC detection of ROS1, MET, and HER2 proteins

Antibodies used for IHC procedures are summarized in Table 3 supplementary data. IHC procedures were performed on 3-µm deparaffinized tissue microarray sections. For antigen retrieval, we used a pH 8.0 EDTA solution (15 minutes of boiling water). IHC against ROS1 protein was performed manually whereas IHC against MET and HER2 was performed on Bond autostainer (Leica Biosystems, Wetzlar, Germany). For ROS1 IHC, HCC78 lung adenocarcinoma and U-138-MG glioblastoma cell lines were used as positive controls. These two cell lines express an SLC34A2-ROS1 fusion and a *FIG-ROS1* fusion, respectively. Mz-ChA1, a gallbladder adenocarcinoma cell line, which is not *ROS1* rearranged and does not express ROS1 protein was used as a negative control. RT-PCR evaluating levels of ROS1 5' and 3' gene regions and anti-ROS1 immunoblotting have been conducted in the laboratory to confirm these data (data not shown). A diffuse cytoplasmic staining was considered as positive. For HER2 IHC, a breast HER2 positive (3+) adenocarcinoma was used as a positive control. A strong complete, basolateral or lateral membranous HER2 staining in more than 10% of tumor cells was considered as strongly positive (equivalent to 3+ score). A weak to moderate complete, basolateral or lateral membranous staining in more than 10% of tumor cells was considered weakly positive (equivalent to 2+ score). Faint or barely perceptible membranous staining in more than 10% (equivalent to 1+ score) and no or staining in less than 10% of tumor cells were both considered negative [33]. For MET IHC, a MET strongly positive lung adenocarcinoma was used as control. Intensity of membranous and cytoplasmic immunostaining was scored according to a four-tier system: no staining (0); weak (1+); moderate (2+); and strong (3+) [34]. Cases with no or weak staining (0 and 1+) were considered negative, and cases with moderate and strong staining (2+ and 3+) were considered positive.

Results

Population

Main clinical characteristics of our series are summarized in Table 1.

One hundred and ninety-four (194) cases of surgically resected biliary tract adenocarcinomas were included in this study. Sex ratio (men to women) was 1.3. Mean age was 65.5 years (SD= 10.9 years) and median age was 66 years (range 29 to 88 years).

Tumor characteristics

Characteristics of tumors according to their location and type are summarized in Table 1.

Fluorescent in situ hybridization results

The chromosomal alterations detected by FISH and the clinical and pathological features associated with those cases are specified in Table 2.

ROS1 chromosomal alterations

In the whole series of 194 biliary tract adenocarcinomas, we did not observe any case of *ROS1* rearrangement or amplification. However, we detected 24 (12.4%) cases with chromosome 6 monosomy or large deletion. For each of these cases, most of tumor cells had only one fused signal of *ROS1* with the *ROS1* Dual Color Break Apart Probe and obvious single *ROS1* and CEP6 signals with the ROS1/CEN 6 Dual Color Probe (Table 2), whereas they contained two *HER2*, *MET* and *ALK* signals. One example of *ROS1* monosomy is shown in Figure 1.

MET amplification

Two of the 194 tumors harbored a *MET* amplification. These tumors were respectively a poorly differentiated intrahepatic cholangiocarcinoma and a well /moderately differentiated perihilar cholangiocarcinoma. Figures 2a, 2b and 2c illustrate the intrahepatic cholangiocarcinoma with *MET* amplification.

ALK alterations

Using the *ALK* corresponding probes, we did not detect any amplification or rearrangement in the whole series.

HER2 amplification

HER2 amplified tumors were more often located in gallbladder adenocarcinoma. Out of the 73 intrahepatic cholangiocarcinomas, using FISH with the *HER2* IQFISH pharmDx™, we detected one case (1.4%) with HER2 amplification (Table 2). This case developed within a mucinous cystic neoplasm and was classified as bile duct type. Out of the 36 distal extrahepatic bile duct cholangiocarcinomas, we detected 2 cases (5.5%) with *HER2* amplification, one developed in an intraductal papillary neoplasm. Out of the 45 gallbladder adenocarcinomas, we detected 5 cases (11.1%) with *HER2* amplification (Table 2), of which 3 were poorly differentiated. None of the 40 perihilar bile duct cholangiocarcinomas harbored *HER2* amplification. An example of a *HER2* amplified tumor is shown in Figures 2d, 2e and 2f.

Unbalanced HER2/CEP17 and ROS1/CEP6 signal ratios and polysomy

Using dual probes with specific probe spanning *HER2* and *ROS1* and their corresponding centromeric probes, we observed three cases (Table 2), with unbalanced ratio of *HER2*/CEP17 and *ROS1*/CEP6, with cell-to-cell variations within tumors. In addition, one of these three cases was also polysomic showing multiple copies of chromosome

6. At last, one case was polysomic with multiple copies of chromosome 17, with a cell-to-cell variation. The percentage of tumor cells with different ratios are summarized in Table 2.

Centromeric alpha-satellite sequences over-representation

We detected five cases with centromeric alpha-satellite sequences over-representation (Table 2). For each of these cases, we observed two specific signals corresponding to *HER2* or *ROS1*, whereas more than 2 centromeric signals were observed in tumor cells. All these cases were intrahepatic cholangiocarcinomas (cholangiolar type n=3, bile duct type n=2).

Correlation between fluorescent in situ hybridization and immunohistochemistry

All cases that carried amplification of *MET* (n=2) or *HER2* (n=8) were positive by IHC. Examples of *MET* and *HER2* positive immunostainings are respectively shown in Figures 2c and 2f.

All non-amplified *HER2* cases were negative by IHC. On the contrary, IHC was positive in 14 (7, 4%) non-amplified *MET* cases. IHC against ROS1 protein did not show any positivity in the whole series (n=194). Cases with unbalanced ratios of *HER2*/CEP17 and *ROS1*/CEP6 chromosomal and case with polysomy showed a negative immunostaining using anti-ROS1, anti-HER2 and anti-MET antibodies.

DISCUSSION

In this series of 194 biliary tract adenocarcinomas, using FISH assays with probes against *ROS1*, *MET*, *ALK* and *HER2* loci, we detected targetable alterations in 5.1% of cases (n=10). From these, 8 cases were *HER2* amplified, while the remaining 2 cases displayed *MET* amplification. The *HER2* amplified cases were mostly gallbladder adenocarcinomas (11.1%). The three other cases were distal extrahepatic and intrahepatic (bile duct type) cholangiocarcinomas, in accordance with other studies [12, 13, 35, 36].

The two cases displaying *MET* amplification were an intrahepatic (bile duct type) and an extrahepatic perihilar cholangiocarcinoma, in accordance with the recent literature suggesting that *MET* amplification is a rare event in biliary tract adenocarcinomas. Indeed, using hybrid capture sequencing, in a large series, Javle et al. detected *MET* amplifications in 2% of intrahepatic cholangiocarcinomas and in 1% of gallbladder adenocarcinomas, whereas none of the 57 extrahepatic bile duct cholangiocarcinomas displayed *MET* amplification [14].

We found no *ROS1* rearrangement in our series of 194 biliary tract adenocarcinomas. This result is in contradiction with the initial studies using a PCR method that reported 8.7 and 9.2% of *ROS1* rearrangements in 23 cholangiocarcinomas and 65 biliary adenocarcinomas respectively [6, 7]. In large series (1.4% of 208 intahepatic cholangiocarcinomas, 1% of 100 cholangiocarcinomas without anatomical or subtype specification), using FISH, with or without an IHC pre-screening, no or very few *ROS1* rearrangement was evidenced, which is in accordance with our findings [8-10]. Therefore, *ROS1* rearrangement is probably a very rare targetable alteration in biliary tract adenocarcinomas.

We did not detect any case of *ALK* rearrangement in our series, which is in accordance with previous studies [9]. We detected *ROS1* monosomy in 13.7% of intrahepatic cholangiocarcinomas, 10% of perihilar cholangiocarcinomas, 16.7% of distal cholangiocarcinomas and 8.9% of gallbladder carcinomas in our series. As we detected one copy of *ROS1* gene and one copy of chromosome 6 centromere, complete loss or extensive deletion of chromosome 6, likely occurred. Partial losses of chromosome 6 have already been described in the literature, but we did not find reports of complete loss [37, 38].

Using FISH and focusing on a subset of target genes, we evidenced four cases with chromosomal alterations with a cell-to-cell variability suggestive of dynamic chromosomal instability. Among these cases, which were mostly gallbladder carcinomas (3/4) two showed unbalanced ratios of *ROS1*/CEP6 and *HER2*/CEP17; one showed both unbalanced *ROS1*/CEP6 and *HER2*/CEP17 ratios and polysomy with multiple copies of chromosome 6 centromere; one showed polysomy with multiple copies of chromosome 17 centromere, without *HER2* over-representation: this last occurrence may also be a pattern of numerical chromosomal instability [41].

Only intrahepatic cholangiocarcinomas (n=5, three cholangiolar and two bile duct type), showed centromeric alpha-satellite sequences over-representation. These sequences are essential elements that stabilize interactions with DNA binding proteins, maintain heterochromatin architecture [42] and over-representation of these sequences seems to be characteristic of some tumors [43]. Such repeats appear to be involved in the development of breast adenocarcinoma [44] and associated with aneuploidy [45], but they have not been reported in biliary tract adenocarcinoma.

Our observations confirm the complexity of genetic abnormalities involved in the process of biliary tract neoplasia, which may underlie the mechanisms of resistance to EGFR inhibitors in cholangiocarcinoma [46, 47]. The balanced rearrangements are probably not the primary or major event, initiating tumor emergence, in cholangiocarcinoma.

The design of the study allowed us to conclude that screening of *HER2* and *MET* amplifications may reliably be performed by immunohistochemistry. IHC seems to be a sensitive and specific method to detect *HER2* amplifications in biliary tract adenocarcinomas. Conversely, MET IHC is a sensitive though not specific method to detect *MET* amplification because 7.3% of the non-amplified MET cases were positive by IHC. Therefore, additional FISH technique is mandatory in MET immunostaining positive cases. The value of IHC to detect *ROS1* or *ALK* rearrangement or amplification, could not be assessed in this study.

The main strength of this study is its large number of cases and the precise anatomical distribution according to WHO and TNM classifications in addition to the main characteristics of the tumors, including their morphological pattern [25, 26, 39]. Indeed, the four types of biliary tract adenocarcinomas have different biological behavior with specific molecular alterations [22, 24]. Therefore, we separated these types in order to obtain accurate data for each type of carcinoma. When analyzing our results by tumor type, we confirm the predominance of *HER2* amplification in gallbladder carcinomas and distal extrahepatic cholangiocarciomas. Centromeric alpha-satellite sequences over-representation seems to concern only intrahepatic cholangiocarcinomas and the frequency of *ROS1* monosomy seems to be quite comparable between the different types of tumor types (8.9% to 16,7%).

Moreover, the design of the study was uniform and systematic: we performed FISH for all targeted chromosomal alterations (*HER2* and *MET* amplifications, *ROS1* and *ALK* rearrangements and amplifications), in our whole series with IHC in parallel. Assessment of IHC was performed blinded to FISH results at the time of evaluation. Because there are currently no official guidelines defining how to assess *HER2*, *MET*, *ROS1* and *ALK* status using FISH and IHC in biliary tract adenocarcinomas, positivity was defined based on well-established criteria, well-known by pathologists, and applied for other organs. Several studies looked for targetable chromosomal alterations in biliary tract adenocarcinomas, but authors often used IHC, RT-PCR, or NGS based methods rather than FISH which is the standard for detecting chromosome rearrangements in pathology laboratories [9, 14, 22, 40]. In some studies, FISH was used, but not systematically which hinders the comparison between the diagnostic tools [9, 10, 18]. In Chiang et al. study, *ROS1* and *ALK* rearrangements were examined using FISH only for tumors over-expressing *ROS1* or *ALK* whereas these authors used IHC only to detect *MET* amplifications, with possible non-amplified IHC positive cases [9].

However, our study has some limitations. First, the small number of tumors with *MET* alterations found (n=2) does not allow a comparison between the tumor types. Second, there is also a low number (n=9) of bile duct type intrahepatic cholangiocarcinomas in our study which leads to the same limitations.

Moreover, our study is monocentric. Therefore, pre-analytical phases, known to influence IHC and FISH assays were comparable for all the samples. On the one hand, this allowed us to investigate these alterations in well-defined experimental conditions. On the other hand, it did not allow us to evaluate the robustness of these techniques between laboratories. The value of IHC for *MET* and *HER2* amplification screening should be confirmed by other studies. Finally, rearrangements have only been studied by FISH which is less informative than RNA sequencing.

To conclude, by studying a subset of target genes in a large series of well-defined biliary tract adenocarcinomas, we confirm the existence of a spectrum of molecular and chromosomal alterations varying with the tumor location, the relatively high rate of *HER2* amplification in gallbladder adenocarcinomas and the efficacy of IHC to screen these cases as to screen *MET* amplification, although very rare in biliary tract adenocarcinomas. *ROS1* rearrangements cannot be regarded as a molecular hallmark of biliary tract adenocarcinomas and, contrary to the initial published data, it seems to be a very rare event. Our data confirm the frequent existence of aneuploidy and chromosomal instability in biliary tract adenocarcinomas and reveal the existence of centromeric alpha-satellite sequences over-representation in intrahepatic cholangiocarcinomas.

Declarations

Funding: This study was funded by the academic grant from AFEF (AAP 2014- R14192DD**).**

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

Availability of data and material (data transparency): on supplementary data. Material is available in AP-HP, Hôpital Saint Antoine, Service d'Anatomie et Cytologie Pathologiques, 75012 Paris, France.

Contributions: Dominique Wendum and Pascale Cervera conceived and designed the study. Jeremy Augustin, Pascale Cervera and Dominique Wendum wrote the manuscript. Pascale Cervera and Jeremy Augustin analysed in situ hybridization data. Jeremy Augustin, Pascale Cervera and Dominique Wendum analysed immunohistochemical data. Françoise Praz supervised RT-PCR and Western blotting experiments. Jean-François Fléjou, Olivier Scatton, François Paye and Françoise Praz edited and reviewed the manuscript. Caroline Gabignon, Aurélie Scriva, Claire Calmel and Laetitia Menu performed and interpreted experiments, and reviewed the manuscript. All authors gave final approval for publication.

Jeremy Augustin, Pascale Cervera and Dominique Wendum take full responsibility for the work as a whole, including the study design, access to data and the decision to submit and publish the manuscript.

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Captions

Table S1: overview of studies on *ROS1, ALK, MET* and *HER2* alterations in biliary tract adenocarcinomas.

Table S2: probes and protocols used for fluorescent in situ hybridization analysis.

Table S3: characteristics of antibodies for the immunohistochemistry analyses.

Table 1: clinical and pathological characteristics of study groups.

Table 2: clinicopathologic details of cases with chromosomal abnormalities and corresponding gene copies number, highlighted by fluorescent in situ hybridization.

Fig. 1. Example of *ROS1* monosomy. Well to moderately differentiated intrahepatic cholangiocarcinoma (**1a**) with only one red green fused signal per cell (**1b**).

Fig. 2 a-c. Example of *MET* amplification. Poorly differentiated intrahepatic cholangiocarcinoma (**2a**); *MET* amplification with more than 6 red signals per cell (**2b**); MET immunohistochemistry showing a positive cell membrane and cytoplasmic staining (**2c**). **d-e** Example of *HER2* amplification. Well to moderately differentiated gallbladder adenocarcinoma (**2d**); *HER2* amplification with more than 15 red *HER2* signals for 2 green centromere signals per cell (**2e**); HER2 immunohistochemistry showing a positive cell membrane staining (**2f**).

Table 1: clinic

	Intrahepa **cholangiolar (n=64)**
	N (%)
Age	
Median	64
Mean (sd)	64.7 (10.6)
Sex	
Male	38 (59.4)
Female	26 (40.6)
Associated risk factors	
None	33 (51.6)
PSC	0 (0)
Mucinous cystic neoplasm	0 (0)
Hemochromatosis	2 (3.1)
Lithiasis	2 (3.1)
MDR3 deficiency	1 (1.6)
Fatty liver disease	9 (12.5)
Alcoholic liver disease	5 (7.8)
HBV	5 (7.8)
HBV+Alcohol	1 (1.6)
HCV	6 (9.4)
HCV+Alcohol	0 (0)
Intraductal papillary neoplasm	0 (0)
Liver fibrosis	
F0	22 (34.3)
F1	9 (14.1)
F2	10 (15.6)
F3	5 (7.8)
F4	13 (20.3)
NA	
NA	5 (7.8)
Tumor size (mm)	
Mean (sd)	70.5 (40.8)
NA	
Multiple tumors	
No	36 (56.2)
Yes	28 (43.8)
T stage *	
T1	21 (32.8)
T2	34 (53.1)
T3	7 (10.9)

T4	1 (1.6)
NA	1 (1.6)
Histologic type	
Cholangiocarcinoma / biliary type adenocarcinoma (NOS)	54 ** (84.4)
Mixed neuroendocrine non-neuroendocrine neoplasm (adenocarcinoma)	0 (0)
Adenosquamous carcinoma	0 (0)
Combined hepatocellular cholangiocarcinoma	9 (14.1)
Lymphoepithelioma-like cholangiocarcinoma	1 (1.6)
Poorly cohesive (with or without signet ring cells)	0
Intestinal type adenocarcinoma	0
Tumor differentiation	
Well or moderate	34 (53.1)
Poor	30 (46.9)
NA	0 (0)
Microvascular invasion	
No	20 (31.2)
Yes	44 (68.8)
Perineural invasion	
No	49 (76.6)
Yes	15 (23.4)
Lymph Node infiltration	
N0	47 (73.4)
N1	14 (21.9)
NA	3 (4.7)
Resection margins	
R0	42 (65.6)
R1	22 (34.4)
NA	0 (0)
Chromosomal abnormalities	
None found	50 (78.1)
HER2 amplification	0
ROS1 translocation	0
ROS1 amplification	0
ALK translocation	0
ALK amplification	0
MET amplification	1 (1.6)
CASOR	3 (4.7)
Chromosomic instability	10 (15.6)
ROS1 monosomy	9 (14)
aneuploidy	1 (1.6)

sd, standard deviation; PSC, primary sclerosing cholangitis; HBV, hepatitis B virus; HCV
* T stage of the 7th TNM UICC classification
** including 2 cholangiolocarcinomas

al and pathological characteristics of study groups

tic (n=73)		Extrahepatic (n=76)	
bile duct type (n=9)		perihilar (n=40)	distal extra hepatic (n=36)
N (%)		N (%)	N (%)
65		68	67
61.8 (10.4)		67.3 (11.4)	65.1 (12.0)
6 (66.7)		19 (47.5)	29 (80.6)
3 (33.3)		21 (52.5)	7 (19.4)
5 (55.6)		39 (97.5)	32 (88.9)
1 (11.1)		0	1 (2.8)
1 (11.1)		0	0
0 (0)		0	0
0 (0)		0	1 (2.8)
1 (11.1)		0	0
0 (0)		1 (2.5)	0
0 (0)		0	1 (2.8)
0 (0)		0	0
0 (0)		0	0
0 (0)		0	0
1 (11.1)		0	0
0 (0)		0	1 (2.8)
5 (55.6)		9 (22.5)	NA
1 (11.1)		9 (22.5)	NA
0		3 (7.5)	NA
1 (11.1)		9 (22.5)	NA
1 (11.1)		4 (10)	NA
1 (11.1)		6 (15)	NA
63.3 (17.8)		30.9 (13.5)	23.8 (11.9)
0		6	8
3 (33.3)		40 (100)	36 (100)
6 (66.7)		0	0
1 (11.1)		2 (5)	3 (8.3)
6 (66.7)		33 (82.5)	8 (22.2)
0		5 (12.5)	25 (69.4)

2 (22.2)		0	0
0		0	0
8 (88.9)		39 (97.5)	36 (100)
0		0	0
0		0	0
1 (11.1)		0	0
0		0	0
0		1 (2.5)	0
0		0	0
6 (66.7)		36 (90)	17 (47.2)
3 (33.3)		4 (10)	19 (52.8)
0 (0)		0 (0)	0 (0)
5 (55.6)		18 (45)	9 (25)
4 (44.4)		22 (55)	27 (75)
6 (66.7)		4 (10)	7 (19.4)
3 (33.3)		36 (90)	29 (80.6)
6 (66.7)		22 (55)	14 (38.9)
3 (33.3)		16 (40)	22 (61.1)
0 (0)		2 (5)	0 (0)
4 (44.4)		25 (62.5)	24 (66.7)
5 (55.6)		15 (37.5)	11 (30.6)
0 (0)		0 (0)	1 (2.8)
5 (55.6)		35 (87.5)	28 (77.8)
1 (11.1)		0	2 (5.6)
0		0	0
0		0	0
0		0	0
0		0	0
0		1 (2.5)	0 (0)
2 (22.2)		0 (0)	0
1 (11.1)		4 (10)	6 (16.7)
1 (11.1)		4 (10)	6 (16.7)
0		0 (0)	0 (0)

V, hepatitis C virus; CASOR, centromeric alpha-satellite sequences over-representation; NA, data nc

Gallbladder (n=45)
N (%)
67
66.1 (11.1)
18 (40)
27 (60)
28 (62.2)
4 (8.9)
0
0
13 (28.9)
0
0
0
0
0
0
0
0
NA
NA
NA
NA
NA
NA
35.3 (19)
15
45 (100)
0
5 (11.1)
14 (31.1)
20 (44.4)

5 (11.1)
1 (2.2)
41 (91.1)
1 (2.2)
1 (2.2)
0
0
1 (2.2)
1 (2.2)
26 (57.8)
18 (40)
1 (2.2)
19 (42.2)
26 (57.8)
24 (53.3)
21 (46.7)
11 (24.4)
18 (40)
16 (35.6)
24 (53.3)
18 (40)
3 (6.6)
33 (73.3)
5 (11.1)
0
0
0
0
0 (0)
0
7 (15.6)
4 (8.9)
3 (6.7)

ɔt available

Table S2: probes and protocols used for fulorescent in situ hybridization analysis

	ROS1 translocation	**ROS1 amplification**
Manufacturer	Zytovision	Zytovision
	ZytoLight ® SPEC ROS1 Dual Color Break Apart Probe	ZytoLight ® SPEC ROS1/CEN 6 Dual Color Probe
Reference	Z-2144-200	Z-2162-200
Digestion	7 minutes	7 minutes
Denaturation	10 minutes; 75°C	10 minutes; 75°C
Hybridization	16 hours; 37°C	16 hours; 37°C
Green spot	6q22.1 proximal	6q22.1
Red spot	6q22.1 distal	alpha-satellites seq on Chr6

cMET amplification	HER2 amplification
Vysis	DAKO
Vysis MET Spectrum Red FISH Probe	HER2 IQFISH pharmDx™
06N05-020	K5731
7 minutes	7 minutes
5 minutes; 73°C	5 minutes; 85°C
16 hours; 37°C	16 hours; 45°C
-	Centromeric region of chromosome 17 (accorgind to manufacturer)
spectrum red 7q31.2	218kb region encompassing HER2 gene (according to manufacturer)

ALK translocation	**ALK amplification**
Zytovision	Zytovision
ZytoLight ® SPEC ALK Dual Color Break Apart Probe	ZytoLight ® SPEC ALK/2q11 Dual Color Probe
Z-2124-200	Z-2161-200
7 minutes	7 minutes
10 minutes; 75°C	10 minutes; 75°C
16 hours; 37°C	16 hours; 37°C
2p23.1 et p23.2	2p23.2
2p23.2 distale	2q11.2

Molecular alteration Meta Analysis in biliary tract adenocarcinomas: PCR, immunohistochemistry, In situ hybridization

Cancer type and localisation	HER2	MET	ALK	ROS	Case number	Authors	journal
intrahepatic cholangiocarcinoma	NR	58% (15)	NR	NR	26	Terada T, Nakanuma Y, Sirica AE (1994)	Hum Pathol;29:175–180.
intrahepatic cholangiocarcinoma	0- NR	21,4%- 0%	NR	NR	28	Nakazawa K, Dobashi Y, Suzuki S, Fujii H, Takeda Y, Ooi A (2005)	J Pathol. ;206:356–365
extrahepatic cholangiocarcinoma	11,5-8,5%	0- NR	NR	NR	78		
Gallbladder adenocarcinoma	15,7-21,6%	0- NR	NR	NR	89		
intrahepatic cholangiocarcinoma	0,009		NR	NR	106	Yoshikawa D, Ojima H, Iwasaki M, Hiraoka N, Kosuge T, Kasai S, Hirohashi S, Shibata T (2008)	Br J Cancer;98:418–425
extrahepatic cholangiocarcinoma	8.5%	NR	NR	NR	130		
adenocarcinomas of the biliary tree (45) and gallbladder (6)	2 (4%)- polysomy 16; Amplification 1/51 (2%)	NR	NR	NR	51	Shafizadeh N, Grenert JP, Sahai V, Kakar S. (2010)	Hum Pathol.; 41(4):485-92.
adenocarcinomas of the extrahepatic biliary tree	0-0	NR	NR	NR	10	Pignochino Y, Sarotto I, Peraldo-Neia C, Penachioni JY, Cavalloni G, et al. (2010)	BMC Cancer 18;10:631
adenocarcinomas of the intrahepatic biliary tree	5 (26,3%)-1 with amplification (5%) and 4 with polysomy (21%)				19		
gallbladder	1(10%)-1 (10%)	NR	NR	NR	10		
	NR	NR	NR	2 FIG-ROS1 fusion	23	Gu T-L, Deng X, Huang F, et al (2011)	PLoS ONE 6:e15640
Gallbladder adenocarcinoma	12,80%	NR	NR	NR	187	Roa I, de Toro G, Schalper K, et al (2014)	Advanced Gallbladder Cancer. Gastrointest Cancer Res
biliary tract carcinomas	NR	NR	NR	FIG-ROS1 fusion : 6 (9%)	65	Peraldo Neia C, Cavalloni G, Balsamo A, et al (2014)	Genes Chromosomes Cancer
intrahepatic cholangiocarcinoma	NR	18 (22%) with high expression			80/110	Chiang N-J, Hsu C, Chen J-S, et al (2016)	Sci Rep. 3;6:25369.
Gallbladder adenocarcinoma	16,6-11,8%	NR	NR	NR	211	Yoshida H, Shimada K, Kosuge T, Hiraoka N.(2016)	Virchows Arch;468(4):431-9
Cholangiocarcinoma	NR	NR	NR	38 (19,1%)-3 (1,1%) intrahépatic	261	Lim SM, Yoo JE, Lim KH, Meng Tai DW, Cho BC, Park YN (2017)	Cancer Res Treat.;49(1):185-192
intrahepatic cholangiocarcinoma	4.8 %-17,9 %	NR	NR	NR	27 with elegible criteria/440	Galdy S, Lamarca A, McNamara MG, et al (2017) Review	Cancer and Metastasis Reviews 36:141–157
extrahepatic cholangiocarcinoma	19.9 %-57.6 %	NR	NR	NR			
						Pellino A, Loupakis F, Cadamuro M, et al (2018) review	Translational Gastroenterology and Hepatology
gallbladder adenocarcinoma	NR	NR	NR	2	14		
extrahepatic cholangiocarcinoma	NR	NR	NR	4	25		
intrahepatic cholangiocarcinoma	NR	NR	NR	0	26		
Gallbladder adenocarcinoma	NR	37 (39,8%)-17 (18,3%)	NR	NR	113	Y. Kim, S. Bang, S. Jee, S. Park, S. Shin, S. S. Paik (2019)	Cancer Res Treat.2019.370
Gallbladder adenocarcinoma	10-10 (5,4%)	NR	NR	NR	186	Albrecht T, Rausch M, Roessler S, et al. (2019)	Virchows Arch. 14 decembre
intrahepatic cholangiocarcinoma	1-1 (micopapillary morphology)	NR	NR	NR	155	Albrecht T, Rausch M, Roessler S, et al. (2019)	BMC Cancer 19:1191
proximal extrahepatic cholangiocarcinoma	2-2	NR	NR	NR	155		
distal extrahepatic cholangiocarcinoma	3-3 (1,4%)	NR	NR	NR	126		

	Location	ROS1 (methods / results)	ALK (methods / results)	MET (methods / results)
Gu et al (2011) [6] PLoS ONE 6 (1):e15640	Not specified (Cholangiocarcinomas) (n=23)	PCR (after immunoaffinity phosphoproteomic screening) FIG-ROS1 fusion 8,7%		
Peraldo Neia et al (2014) [7] Genes Chromosomes Cancer 53 (12):1033-1040	gallbladder carcinoma (n=14)	PCR FIG-ROS1 fusion 14,3%		
	extrahepatic cholangiocarcinoma (n=25)	PCR FIG-ROS fusion 16%		
	intrahepatic cholangiocarcinoma (n=26)	PCR FIG-ROS fusion 0		
Lim et al (2017) [8] Cancer research and treatment 49 (1):185-192	extrahepatic cholangiocarcinoma (n=53)	FISH (break apart) 0 (n=53)		
	intrahepatic cholangiocarcinoma (n=208)	FISH (break apart) 1,4%		
Chiang et al (2016) [9] Scientific reports 6:25369.	gallbladder carcinoma + extrahepatic cholangiocarcinoma (n=30)	FISH (pre-screening by IHC) 0	FISH (pre-screening by IHC) 0	IHC 0
	intrahepatic cholangiocarcinoma (n=80)	FISH (pre-screening by IHC) 0	FISH (pre-screening by IHC) 1,2%	IHC 7,5%
Graham et al (2014) [10] Human Pathology 45 (8):1630-1638	Cholangiocarcinoma Anatomical location not systematically specified (n=100)	FISH (break apart) 1%		
Voss et al (2013) Human Pathology 44: 1216-1222	intrahepatic cholangiocarcinoma (n=67)			MassARRAY (mutation) 4,5%
	extrahepatic cholangiocarcinoma (n=27)			3,70%
Nakazawa et al (2005) J Pathol. ;206:356–365	gallbladder carcinoma (n=89)			FISH (pre-screening by IHC) 0

	extrahepatic cholangiocarcinoma (n=78)			0
	intrahepatic cholangiocarcinoma (n=28)			0
Yoshikawa et al (2008) British Journal of cancer 98; 418-425	extrahepatic cholangiocarcinoma (n=130)			
	intrahepatic cholangiocarcinoma (n=106)			
Shafizadeh et al ((2010) Human Pathology 41: 485-492	gallbladder carcinoma (n=6)			
	extrahepatic cholangiocarcinoma (n=19)			
	intrahepatic cholangiocarcinoma (n=26)			
Yoshida et al (2016) Virchows Archivs;468(4):431-9	gallbladder carcinoma (n=211)			
Albrecht et al (2019) BMC Cancer 19:1191	distal extrahepatic cholangiocarcinoma (n=126)			
	perihilar extrahepatic cholangiocarcinoma (n=155)			
	intrahepatic cholangiocarcinoma (n=155)			
Albrecht et al (2019) Virchows archiv doi: 10.1007/s00428-019-02706-6.	gallbladder carcinoma (n=186)			

Kim et al. (2019) Cancer Res Treatment doi: https://doi.org/10.4143/crt.2019.370	gallbladder carcinoma (n= 93)			IHC (overexpression) 39,8% FISH (amplification) 9.7 to 18;3% according to the interpretation rules
Javle et al (2016) [32] Cancer 122 (24) : 3838-47	gallbladder carcinoma (n=85)			1%
	extrahepatic cholangiocarcinoma (n=57)			0
	intrahepatic cholangiocarcinoma (n=412)			2%

HER2 **(methods / results)**
FISH (pre-screening by IHC) 3% (2 extrahepatic, 1 intrahepatic)
FISH (pre-screening by IHC) 10%

4%
0
IHC 8.5%
IHC 0.9%
IHC 0
10,5%
0
FISH (pre-screening by IHC) 16,6%
FISH (pre-screening by IHC) 2,4%
1,30%
0,60%
FISH (pre-screening by IHC) 5,4%

Next-generation sequencing 16%
11%
3%

Table S2: probes and protocols used for fulorescent in situ hybridization analysis

	ROS1 translocation	**ROS1 amplification**
Manufacturer	Zytovision	Zytovision
	ZytoLight ® SPEC ROS1 Dual Color Break Apart Probe	ZytoLight ® SPEC ROS1/CEN 6 Dual Color Probe
Reference	Z-2144-200	Z-2162-200
Digestion	7 minutes	7 minutes
Denaturation	10 minutes; 75°C	10 minutes; 75°C
Hybridization	16 hours; 37°C	16 hours; 37°C
Green spot	6q22.1 proximal	6q22.1
Red spot	6q22.1 distal	alpha-satellites seq on Chr6

cMET amplification	HER2 amplification
Vysis	DAKO
Vysis MET Spectrum Red FISH Probe	HER2 IQFISH pharmDx™
06N05-020	K5731
7 minutes	7 minutes
5 minutes; 73°C	5 minutes; 85°C
16 hours; 37°C	16 hours; 45°C
-	Centromeric region of chromosome 17 (accorgind to manufacturer)
spectrum red 7q31.2	218kb region encompassing HER2 gene (according to manufacturer)

ALK translocation	**ALK amplification**
Zytovision	Zytovision
ZytoLight ® SPEC ALK Dual Color Break Apart Probe	ZytoLight ® SPEC ALK/2q11 Dual Color Probe
Z-2124-200	Z-2161-200
7 minutes	7 minutes
10 minutes; 75°C	10 minutes; 75°C
16 hours; 37°C	16 hours; 37°C
2p23.1 et p23.2	2p23.2
2p23.2 distale	2q11.2

Table S3: characteristics of antibodies for the immunohistochemistry analyses

	ROS1	MET	HER2
Manufacturer	Cell Signaling Technology	Bio SB	Dako
Clone reference	monoclonal D4D6	monoclonal BSB 6588	polyclonal A0485
Species	rabbit	rabbit	rabbit
Dilution	1/50	1/200	1/400
Incubation	overnight	20 minutes	20 minutes