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Original article

Morpho-molecular approach (NGS *plus* digital PCR) in diagnosis of malignant biliary strictures

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Summary

Objective. To analyze the diagnostic accuracy and feasibility of *digital*-PCR (dPCR) combined with next-generation sequencing (NGS) in the ERCP-guided histological diagnosis of biliary strictures to overcome the issue represented by the scarcity of sampled material.
Methods. Twenty-two prospective patients were included, and submitted to ERPC-guided biopsy or biliary resection. By histopathological analysis plus fluorescence in situ hybridization (FISH) for chromosomes 3, 7, and 17 aneuploidies, 8 cases (36.4%) were malignant, and 14 cases (63.6%) were negative. NGS was performed on paraffin-embedded tissue by a laboratory-developed panel allowing the analysis of hot-spot regions in 28 genes. Digital PCR (dPCR) was performed by QuantStudio™ AbsoluteQ™ solid dPCR and the copy-number variation (CNV) of the chromosomes 3, 7, and 17 analysed.
Results. At dPCR, 1 case showed aneuploidy of chromosome 3, and 2 cases of both chromosomes 3 and 7. These 3 cases all belonged to the positive group ($p = 0.014$). At NGS, 6 cases showed at least one mutated gene, all in the positive group ($p < 0.001$). The 3 cases showing aneuploidy at dPCR also showed mutations at NGS. Basing on these observations, we can propose a diagnostic algorithm: dPCR can be applied first, allowing a diagnosis of malignancy in one working day if aneuploidies are observed. In the case of negative dPCR, a "second-line" NGS is performed on the same extracted material.
Conclusions. The implementation of dPCR allowed the identification of nearly 40% of positive cases in just one working day. In cases of negative dPCR, the NGS procedure can start on the same extracted nucleic acid used for dPCR, requiring more time, but reaching a 75% sensitivity. More studies are required to identify other more sensitive and specific dPCR targets, but even if our algorithm does not increase diagnostic accuracy, the possibility of avoiding FISH and reaching a diagnosis in a more time- and money-saving fashion might be an important step.

Key words: bile duct carcinoma, biliary stricture, digital polymerase chain reaction, next-generation sequencing

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Introduction

Biliary tract cancers (BTC) are a heterogeneous group of malignant neoplasms including intrahepatic cholangiocarcinoma (CCA), perihilar CCA, distal CCA and gallbladder cancer[1]. BTCs are characterized by a relatively low incidence in Western Countries, but also by an extremely poor prognosis, due to the late diagnosis and the poor response to standard therapies[2]. Bile tract stenosis can be the first manifestation of BTC, but also of benign conditions, such as primary sclerosis cholangitis (PSC), and secondary cholangitis (infective, ischemic, congenital, etc.)[3].

In Western Countries, PSC is the main risk factor for both biliary stenosis and BTC. Most patients with PSC eventually develop a dominant stricture, radiologically defined as a reduction ≤1.5 mm of the diameter of the common biliary duct, or ≤1 mm in a hepatic duct within 2 cm from the main confluence[4,5]. A PSC patient with a dominant stricture has more than 26% increased risk of developing CCA[4]. Thus, the differential diagnosis between benign and malignant biliary strictures is crucial, especially in front of the need of performing a liver transplant to treat PSC, which would be contraindicated in case of malignancy[6]. An additional issue is represented by the high incidence of post-operative complications of biliary surgery[7], making crucial an appropriate selection of those patients who would benefit best from resection: a single study reported that more than 25% of cases initially labeled as malignant proved to be non-neoplastic at post-operative analysis[8].

Cholangio-magnetic resonance is the gold-standard technique for the biliary tract, able to detect strictures in nearly all cases, but with low specificity in differentiating benign versus malignant strictures[9]. Thus, recent guidelines suggested endoscopic retrograde cholangiopancreatography (ERCP) with the execution of cyto-brushing and/or biopsy (if technically possible) for a pathological diagnosis[5,10]. To improve diagnostic accuracy, the guidelines of the World Health Organization (WHO) suggest placing fluorescence *in situ* hybridization (FISH) alongside morphology, with a gain in sensitivity up to 80% and 100% specificity[11]. In particular, polysomies in centromeric regions of chromosomes 3, 7, and 17, as well as homozygous deletions of the 9p21 locus (*CDKN2A* gene) are considered the most important to diagnose malignancy[12]. However, FISH is expensive and time-consuming, requires a specific laboratory and technical competence, and it always requires a suspect morphology, since some pre-neoplastic conditions (e.g. BilIN) can result positive[13].

For all these reasons, some clinical centers have started to apply Next-Generation Sequencing (NGS) as the main (and frequently sole) ancillary technique for bile duct diagnostics: according to some authors, the finding of driver mutations on bile duct material has a sensitivity towards CCA diagnosis similar to fluorescence in situ hybridization (FISH)[10,14]. NGS has its limitations as well, primarily represented by the need of sufficient tumour cells for the analysis: at least 10% of lesional cell enrichment is required, and a failure rate of nearly 27% was originally estimated[15].

The aim of the present paper was to apply a different diagnostic algorithm for bile duct biopsies, adding the digital polymerase chain reaction (dPCR) technique. The dPCR was applied to determine the presence of copy-number variation (CNV) of chromosomes 3, 7, and 17 using specific probes. Our final goals were: (i) to identify an ancillary technique more feasible than FISH as well as more time- and cost-effective than NGS; (ii) to propose an alternative diagnostic algorithm for biliary stenosis, in order to make the best possible use of the scarce material available.

Materials and methods

Ethics and Patients Enrolment

The present study received the approval of the Ethics Committee of the Area Vasta Emilia Centrale (AVEC), protocol number 128/2023/Sper/AOUBo. Patients signed informed consent for the study, and were kept anonymous. This study follows the guidelines of the 1975 Declaration of Helsinki (and following amendments).

All consecutive patients undergoing a bile duct biopsy in 2023 were prospectively enrolled. For the purposes of the study, two kinds of material were analyzed: (I) tissue from ERCP-guided biopsies from patients with primary biliary strictures, and (II) surgical margins from the biliary tree in patients resected for suspect of CCA, and sent for frozen-section analysis. These two types of materials have in common the paucity of cells that sometimes make histopathological diagnosis difficult (and therefore usually require ancillary tests). Inclusion criteria were the retrieval of sufficient material for histopathological and molecular analysis, age ≥18 years, and signed informed consent. Clinical data collected included: PSC or other biliary risk factors, serum CA19.9 levels, and association with inflammatory bowel disease.

Histopathological analysis

Tissue from the biliary tree retrieved during ERCP was sent to the Pathology Unit, formalin-fixed and paraffin embedded (FFPE), and routinely processed. From paraffin blocks, 2 µm thick sections were cut for routine hematoxylin-eosin staining, together with 4 µm thick sections for FISH, and four-to-five 10 µm thick sections for NGS and dPCR.

According to histopathology, cases were classified into three groups (Fig. 1):

- Positive cases: unequivocal presence of an infiltrating carcinoma.
- Negative cases: absence of morphological criteria for carcinoma.
- Doubtful cases: cases in which the qualitative or quantitative limits of the material could suggest a diagnosis of malignancy, but without reaching criteria of certainty. These cases were further analyzed by FISH, according to the current guidelines[11].

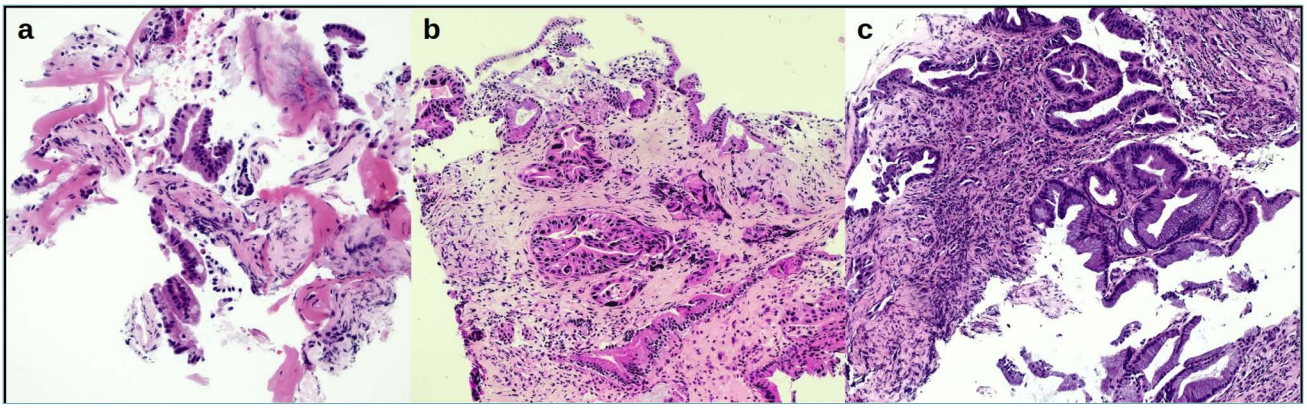

Figure 1. (a) A negative case, with few bland benign-looking glands. (b) A positive case, with infiltrating e glands and cells with high nuclear-to-cytoplasmic ratio and nuclear atypia. (c) A case considered doubtful in our series, lacking full criteria for a diagnosis of malignancy. Hematoxylin-eosin staining, magnification 200x.

Fluorescence in situ Hybridization

FISH analysis was performed on FFPE 3-4 µm thick tissue sections. Once dewaxed overnight at 56°C, the sections were washed 3 times (10 minutes each) with xylene, to remove the paraffin (de-paraffinization). The xylene was then removed by 2 washes (10 minutes each) with absolute ethanol. The sections were placed in a 77°C 2x SSC (saline-sodium citrate) buffer and then moved to a 47°C proteinase K working solution for tissue and protein digestion. Finally, the sections were dehydrated in 1-minute steps through graded concentrations of ethanol, 70% - 96% - 100%, and air-dried at room temperature. Copy number (CN) of chromosomes 3, 7, and 17 was detected by using the ZytoLight CEN3 SpectrumOrange Probe (ZytoVision, Bremerhaven, Germany) targeting sequences mapping in 3q11.1-q11.1, the CEP 7 Probe (Vysis-Abbott, Chicago, Illinois, USA) labeled with SpectrumGreen and hybridizing to the 7q11.1-q11.1 centromeric region and the CEN17 SpectrumGreen Probe TITAN FISH Probe (OACP IE LTD, Cork, Ireland) targeting the 17q12 region. The hybridization area covered with a few microliters of the probe was cover-slipped, sealed with rubber cement, and subjected to denaturation and subsequent hybridization overnight, according to the manufacturer's guidelines. Slides were then rinsed in 2x SSC solution and washed in NP-40 0.3%/2xSSC at 67°C for 1 min for CEN3 and CEN17 probes or at 73°C for 2 min for CEP7 probe. Finally, the slides were dehydrated and mounted by using a DAPI counterstaining solution (Leyca System).

The staining performance and the count of the hybridization signals were performed by using the Olympus BX61 fluorescence microscope (Evident Corporation, Japan) and the Cytovision Image analysis software (CytoVision®). At least 60 nuclei exhibiting signals for each probe were evaluated for each target. According to previously reported cut-off[16,17], specimens showing hybridization patterns as ≥ 5% of cells with more than 5 signals, ≥ 5% of cells with more than 4 signals, and ≥ 4% of cells with more than 3 signals were considered as positive for chromosome 3, 7 or 17 aneuploidies, respectively.

Specimens showing hybridization patterns reaching the cut-off values for at least two chromosomes were then designated as positive[16,17].

Next-generation Sequencing

DNA was extracted starting from three to four 10 µm–thick sections, using the "QuickExtract™ FFPE DNA Extraction Solution" kit (Lucigen Corporation, Middleton, WI, USA), and quantified by Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Considering the kind of lesions, the whole area of biopsies was used for the DNA extraction. About 10 ng of gDNA was used for the library preparation. Libraries were set up using a multi-gene panel developed in the Molecular Pathology Laboratory, IRCCS Policlinico di S. Orsola, Bologna, Italy. The panel allows the analysis of the following hot-spot regions or coding sequencing (CDS) of 20 genes, for a total of 567 amplicons (human reference sequence hg19/GRCh37, 43.03 kb) in the following genes: *ARID1A* (CDS), *BAP1* (CDS), *BRAF* (Exons 11, 15), *CDKN2A* (CDS), *CTNNB1* (Exons 3, 7, 8), *FGFR1* (CDS), *FGFR2* (CDS), *FGFR3* (CDS), *FGFR4* (CDS), *GNAS* (CDS), *HRAS* (Exons 2, 3, 4), *IDH1* (Exon 4), *IDH2* (Exon 4), *KRAS* (Exons 2, 3, 4), *NRAS* (Exons 2, 3, 4), *POLE* (CDS), *SMAD4* (CDS), *STK11* (CDS), *TERT* (promoter region, g.1295171-1295375), *TP53* (CDS).

The results were analyzed using the Ion Reporter software (version 5.20, Thermo Fisher Scientific) and the Golden Helix GenomeBrowse sequencer viewer (version 3.0, https://www.goldenhelix.com/products/GenomeBrowse/index.html). The prediction of the significance of the detected mutations was performed using the Varsome database (https://varsome.com/, last access 29th August 2024). Only single nucleotide variants/indels present in at least 5% of the generated reads and observed in both strands were considered for variant calling[18].

Digital Polymerase-Chain Reaction

The extracted DNA was also used for solid digital Polymerase-Chain Reaction analysis (dPCR). dPCR was performed by QuantStudio™ Absolute Q™ Digital PCR System (Thermo Fisher Scientific). The following probes (Thermo Fisher Scientific) were used to determine the presence of copy-number variation (CNV) of chromosomes 3, 7, and 17:

- chromosomes 3: *CADM2* (3p12.1 – FAM, Hs01672831) and *ZPLD1* (3q12.3 – ABY, Hs03043199)
- chromosomes 7: *EGFR* (7p11.2 – FAM, Hs07526740) and *AUTS2* (7q11.22 – ABY, Hs03643600)
- chromosomes 17: *EPN2* (17p11.2 – ABY, Hs03956962) and *ERBB2* (17q12 – FAM, Hs00817646)

TaqMan Copy number Reference Assay RNase P (14q11.2, Thermo Fisher Scientific) was used as "internal control". The PCR program did not need to be adapted for the analysis: 96°C for 10 min, followed by 40 cycles of 96°C for 5 s and 60°C for 15 s. Data were analyzed using Quant Studio Absolute Q Digital PCR software (v. 6.3). A [chromosome probe/internal control probe] ratio less than 1 was considered a "loss", while a ratio greater than 4 was a "gain". If the [p-arm/q-arm] ratio was equal to 1 (±0.5) the CNV was considered an aneuploidy (monosomy or polysomy), if not, the CNV was considered as "partial LOH" (i.e., not the whole chromosome was unbalanced).

Statistical Analysis

Statistical analysis was carried out with the Jamovi software for Windows, ver. 2.3 (The jamovi project (2022). [Computer Software]. Retrieved from https://www.jamovi.org). Variables are reported as means±standard deviations, ranges and frequencies. Chi-squared (χ^2) test, followed by Fisher's exact test, were applied to compare variables, using a p coefficient < 0.05 to exclude the null hypothesis. Sensitivity and specificity with 95% confidence interval (CI), as well as positive predictive value (PPV) and negative predictive value (NPV), were calculated using the histopathological (plus FISH) diagnosis as gold standard.

Results

Clinical and pathological data

Twenty-three patients were enrolled, 15 (65.2%) males and 8 (34.8%) females, mean age 69±13 years (range 37-91 years). All baseline clinical and pathological characteristics are summarized in Table I: biopsy material came from ERCP-guided biopsies in 13 (56.5%) patients, and from surgical margins (sent for frozen-section analysis during surgery) in 10 (43.5%); all patients presented an increase in serum bilirubin; 12 (52.1%) patients showed elevated CA19-9 levels (over 40 U/mL), 4 (17.4%) had a previous diagnosis of PSC, 10 (43.5%) had cholelithiasis or other chronic cholestatic diseases; 15 (65.2%) had a chronic hepatic disease; one patient had a concomitant ulcerative colitis.

No correlations were observed between the clinical features (including stenosis localization and associated conditions), conclusive diagnosis and the subsequent molecular analyses (data not shown).

One patient was immediately excluded from the analyses, due to a scarcity of sampled cells (less than 100) preventing the execution of FISH, NGS and dPCR. The sample was a specimen with some inflammatory cells and few epithelial cells (less than 50). As previously stated, at least 60 nuclei have to be enumerated for FISH evaluation. Considering that at least 10 ng of DNA was used for NGS library preparation, it means that the total number of cells used for extraction procedures should be at least 1000 and then the specimen was considered as not evaluable for the NGS analysis. The starting material should be sufficient for dPCR (at least 1 copy of DNA/ul) but it was not performed as two of the three techniques being compared in the study did not meet the threshold for evaluability.

At histopathological analysis, the cases were diagnosed as follows:

- Morphologically positive: 5 cases (22.7%), i.e. 4 biopsies and 1 surgical margin.
- Morphologically negative: 11 cases (50.0%), i.e. 4 biopsies and 7 surgical margins.
- Morphologically doubtful: 6 cases (27.3%), i.e. 4 biopsies and 2 surgical margins.

The 6 doubtful cases were further analyzed by FISH (see also Fig. 2 for the diagnostic algorithm): 3 cases showed aneuploidy and 3 did not, so the final diagnosis of our cases was:

Table I. Overview of the baseline clinical and pathological characteristics of the studied cases.

Case	Sex	Age	Sample Type	PSC	Other Cholestatic Disease	Chronic Liver Disease	Elevated CA19.9	IBD	High Serum Bilirubin	Location	Diagnosis (Histo + FISH)	NGS	dPCR Chr3	dPCR Chr7	dPCR Chr17
1	M	62	Biopsy	Yes	Yes	Yes	No	No	Yes	Common bile duct	Negative	WT	Neg	Neg	Neg
2	M	80	Biopsy	No	No	No	Yes	No	Yes	Common hepatic duct	**Positive**	**CDKN2A, SMAD4, TP53**	Neg	Neg	Neg
3	F	65	Biopsy	No	No	No	Yes	No	Yes	Right+Left hepatic ducts	Negative	WT	Neg	Neg	Neg
4	M	76	Biopsy	No	Yes	No	N/A	No	No	Common bile duct	**Positive**	WT	Neg	Neg	Neg
5	M	62	Surgical Margin	No	No	No	Yes	No	Yes	Right+Left hepatic ducts	**Positive**	**TP53**	**CNV**	**CNV**	Neg
6	M	85	Biopsy	No	Yes	No	Yes	No	Yes	Common bile duct	**Positive**	**ARID1A, CTNNB1, TP53**	**CNV**	**CNV**	Neg
7	M	85	Biopsy	No	Yes	No	Yes	No	Yes	Common bile duct	**Positive**	**BRAF**	Neg	Neg	Neg
8	F	91	Biopsy	No	No	No	N/A	No	Yes	Common bile duct	**Positive**	**KRAS, TP53**	**CNV**	Neg	Neg
9	M	66	Surgical Margin	No	Yes	Yes	No	No	Yes	Common bile duct	Negative	WT	Neg	Neg	Neg
10	F	65	Biopsy	Yes	Yes	No	N/A	No	Yes	Common bile duct	Negative	WT	Neg	Neg	Neg
11	M	72	Biopsy	No	Yes	No	No	No	Yes	Common bile duct	**Positive**	**KRAS, TP53**	Neg	Neg	Neg
12	F	72	Biopsy	No	Yes	No	Yes	No	Yes	Common bile duct	Negative	N/A	N/A	N/A	N/A
13	F	70	Biopsy	No	No	No	Yes	No	No	Right+Left hepatic ducts	Negative	WT	Neg	Neg	Neg
14	M	80	Surgical Margin	No	Yes	No	Yes	No	Yes	Common hepatic duct	Negative	WT	Neg	Neg	Neg
15	M	65	Surgical Margin	No	No	Yes	Yes	No	Yes	Common hepatic duct	Negative	WT	Neg	Neg	Neg
16	M	51	Surgical Margin	Yes	Yes	Yes	No	No	Yes	Common bile duct	Negative	WT	Neg	Neg	Neg
17	F	51	Surgical Margin	Yes	No	Yes	N/A	Yes	Yes	Common bile duct	Negative	WT	Neg	Neg	Neg
18	M	64	Surgical Margin	No	Yes	Yes	Yes	No	Yes	Common bile duct	Negative	WT	Neg	Neg	Neg
19	F	37	Surgical Margin	No	No	No	No	No	Yes	Common bile duct	**Positive**	WT	Neg	Neg	Neg
20	M	53	Surgical Margin	No	No	No	Yes	No	Yes	Common bile duct	Negative	WT	Neg	Neg	Neg
21	M	79	Biopsy	No	Yes	No	No	No	Yes	Common bile duct	Negative	WT	Neg	Neg	Neg
22	M	77	Surgical Margin	No	Yes	No	No	No	Yes	Right+Left hepatic ducts	**Positive**	WT	Neg	Neg	Neg
23	M	80	Biopsy	No	No	Yes	Yes	No	No	Common bile duct	Negative	WT	Neg	Neg	Neg

- Positive cases: 8 cases (36.4%), i.e. 5 biopsies and 3 surgical margins.
- Negative cases: 14 cases (63.6%), i.e. 7 biopsies and 7 surgical margins.

Digital Polymerase-Chain Reaction and Next-Generation Sequencing: proposal of a diagnostic algorithm

At dPCR, 1 case showed aneuploidy of chromosome 3, and 2 cases showed aneuploidy of both chromo-

some 3 and 7 (Tab. II). These 3 cases all belonged to the positive group (p = 0.014, χ^2 test; p = 0.036, Fisher's exact test; Cohen's *Kappa* 0.433 – moderate agreement). dPCR showed 100% specificity (95% CI: 76.8%-100.0%) toward the diagnosis of malignancy, in front of 37.5% sensitivity (95% CI: 8.5%-75.5%), with a PPV of 100% and a NPV of 73.7% compared to the *gold standard*.

Table II. dPCR results: 3 out of 8 (37.5%) positive cases showed at least one aneuploid chromosome among those analyzed, with aneuploidy in Chromosome 3 always present.

dPCR		
	Nr.	**Aneuploidy**
Negative cases	14	None
Positive cases	8	5 *No aneuploidies found* 2 *Chrom.3 and Chrom. 7* 1 *Chrom. 3*
Not assessable	1	N/A
Total	23	

At NGS, 6 cases showed at least one mutated gene, all among the positive group, so that NGS sensitivity was 75.0% (95% CI: 29.0%-96.3%). Specifically, 2 cases showed mutations in *KRAS* and *TP53*, 1 case in *BRAF*, 1 case in *ARID1A* and *TP53*, 1 case in *BRAF*, and 1 case in *CDKN2A*, *SMAD4* and *TP53* (Tab. III). All negative cases were wild type for all the evaluated genes, so that NGS specificity was 100% (95% CI: 75.3%-100.0%).

The association between morphological-FISH diagnosis and NGS was statistically significant ($p < 0.001$, χ^2 test; $p < 0.001$, Fisher's exact test; Cohen's *Kappa* 0.765 – substantial agreement). NGS showed a PPV of 100% and a NPV of 86.7% compared to the *gold standard*.

Table III. NGS results: 6 out of 8 (75.0%%) positive cases showed mutations in at least one of the genes analyzed, with mutations in *TP53* associated in most cases.

NGS		
	Nr.	**Mutation**
Negative	14	None
Positive	8	2 *wild type* 2 *KRAS* + *TP53* 1 *ARID1A* + CTNNB1 + *TP53* 1 *CDKN2A* + *SMAD4* + *TP53* 1 *BRAF* 1 *TP53*
Not assessable	1	N/A
Total	23	

The 3 cases showing aneuploidy at dPCR also showed mutations at NGS. In detail, dPCR showed a 40% sensitivity towards NGS (95% CI: 5.3%-85.3%), with 100% specificity (95% CI: 78.2%-100.0%). Based on these observations, we can propose a diagnostic algorithm, represented in Figure 2: dPCR can be applied first, allowing a diagnosis of malignancy in one working day if aneuploidies are observed. In the case of negative dPCR, a "second line" NGS is performed on the same extracted material.

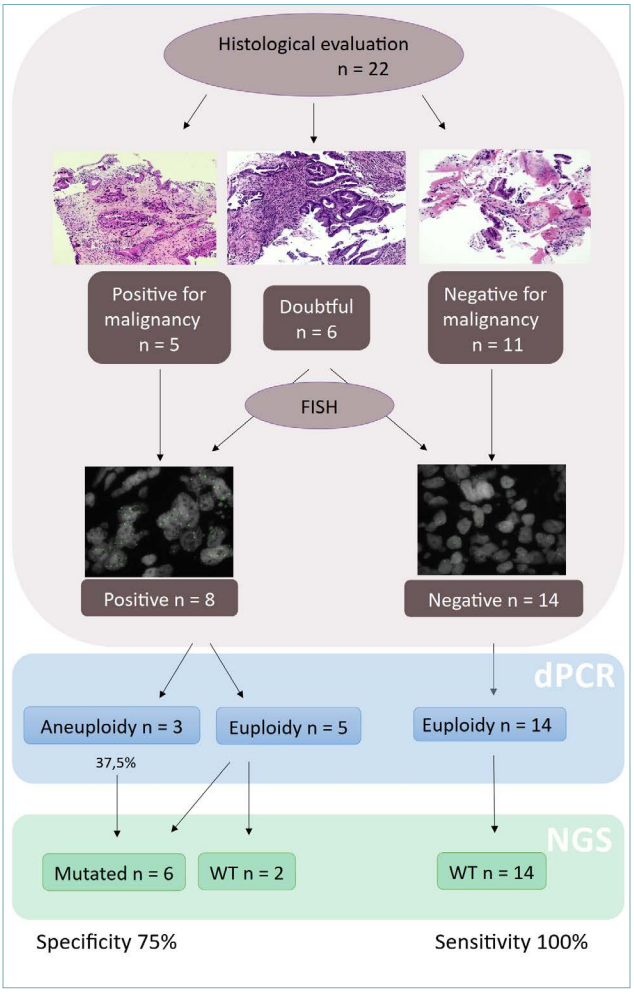

Figure 2. The diagnostic algorithm proposed according our results: dPCR can replace FISH, saving time (just 1 working day needed) and lab resources, despite the relatively low sensitivity. In the cases negative at dPCR, NGS should be carried out, using the same extracted material. Please note: in one case of our series, the extracted material was not suitable for either methodologies.

Discussion

The differential diagnosis of biliary strictures in patients with chronic bile duct diseases (PSC above all) is still a challenge. When a radiological dominant stricture is found, the most recent guidelines suggest the execution of ERCP followed by biopsy or cytobrushing[5,6,10]. Nevertheless, in front of such an invasive and technically difficult procedure, the retrieved tissue material is very frequently scarce, artifactuall, and lacking many criteria for a comfortable diagnosis of malignancy. To overcome this problem, the WHO and other guidelines strongly suggest the execution of FISH, the presence of aneuploidy in chromosomes 3, 7, 17 being the strongest indication for high-grade dysplasia/adenocarcinoma[7,12-14]. In the current setting, due to the centralization of many healthcare structures in Italy and other countries, a FISH lab is not always available, requiring specific spaces, technical competences and procedures. In increasingly more institutions, those resources are spent on other methodology, primarily NGS and other extractive techniques. This is why NGS is now suggested as primary support to histopathological analysis in the diagnosis of BTC, and it is definitely set to become the strongest methodology in the future, for research as well as for routine applications[14,19,20]. Nonetheless, NGS is not free from limitations, mainly represented by the longer technical times, and the need for sufficient tumor cells in the specimen, which is precisely the main issue of pancreato-biliary pathology.

The aim of the present work was to propose a pathological and molecular diagnostic algorithm, alternative to FISH. The implementation of dPCR satisfied our expectations, allowing the identification of nearly 40% of positive cases in a very short time (1 working day, just 2 hours after DNA extraction). In cases of negative dPCR, the NGS procedure starts on the same extracted material, increasing the time to 3 working days, but reaching 75% sensitivity, (comparable to FISH)[11,12,20]. This is a preliminary series, which needs validation in a prospective cohort. Moreover, the choice of analysing chromosomes 3, 7, and 17 with dPCR was arbitrary, mirroring the main targets of FISH analysis. This does not preclude other more sensitive and specific dPCR targets from being proposed in future studies. Even if our algorithm does not increase diagnostic accuracy *per se*, we think that the possibility of avoiding FISH and reaching a diagnosis in a more time- and resource in one-saving fashion might be an important step.

Conflicts of interest statement

The Authors have no conflicts of interest to declare.

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Authors' contributions

FV: study design, data analysis, funding, paper drafting; EA and LM: data collection and analysis; TM, SCo: methodology; SCh: data analysis; GT, ADE: critical revision; DdB: conceptualization, critical revision, paper drafting.

Ethical considerations

The present study received the approval of the Ethical Committee of the Area Vasta Emilia Centrale (AVEC), protocol number 128/2023/Sper/AOUBo. Patients signed informed consent for the study, and they are kept anonymous. This study follows the guidelines of the 1975 Declaration of Helsinki (and following amendments).

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