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Analysis of microRNA expression in brush cytology specimens improves the diagnosis of pancreatobiliary cancer

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ABSTRACT

Background/Objectives: Malignant pancreatobiliary strictures are in many cases clinically indistinguishable and present a major problem to endoscopy specialists. Intraductal sampling procedures such as brush cytology are commonly used for diagnosis with a sensitivity that is low for a diagnostic test used in daily clinical practice. MicroRNA (miR) alterations detected in many cancers are disease-specific, which can be utilized in clinical applications. The aim of the present study was to analyze whether determination of miR expression levels in intraductal brush cytology specimens is a feasible approach to improve the diagnosis of pancreatobiliary cancer.

Methods: Brush cytology specimens have been collected during endoscopic retrograde cholangiopancreatography (ERCP) and analyzed by routine cytology and ancillary miR assays. Total RNA was extracted using the miRNeasy Mini Kit and the expression of miRs frequently dysregulated in pancreatobiliary cancer (miR-16, miR-21, miR-196a, miR-221) were analyzed by quantitative real-time PCR using RNU6B as internal control.

Results: Routine cytology resulted in no false positive diagnoses, however, the combined sensitivity remained at 53.8%. Expression (ΔCt values) of miR-16 ($p = 0.0039$), miR-196a ($p = 0.0003$) and miR-221 ($p = 0.0049$) showed a clear statistical significance between malignant and benign pancreatobiliary specimens ($n = 35$). Malignancy could be detected combining routine cytology and the miR-196a single marker expression levels with a sensitivity of 84.6% (92.9% in biliary strictures) with no false positives.

Conclusions: The results offer the first direct demonstration that microRNAs are readily detectable in brush cytology specimens obtained during ERCP, and have the potential to help the cytological diagnosis of pancreatobiliary malignancy.

Introduction

Pancreatic cancer is expected to become the second leading cause of cancer-related death by 2030 surpassing breast, prostate, and colorectal cancers [1]. Pancreatobiliary malignant strictures are hard to diagnose, manage, and often manifest in advanced unresectable stages. Early diagnosis and precise staging are hindered by the lack of specific symptoms, biomarkers and the shortcomings of cross-sectional imaging [2,3].

ERCP-based brush cytology is widely available, because it is a cheap, fast, relatively easy to perform and a safe procedure with a specificity approaching 100%. In a recent meta-analysis the pooled sensitivity and specificity of brushings for the diagnosis of malignant biliary strictures was 45% and 99% respectively, even in a combination with intraductal biopsies the sensitivity only reached 59.4% [4]. The low sensitivities measured in several studies [5], combined with the availability of more recent diagnostic procedures such endoscopic ultrasound (EUS), have led to questions over

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the utility of ERCP brush cytology in diagnosing pancreatobiliary disease. ERCP is still the only methodology that can treat the jaundiced and yield a cytology diagnosis in a single procedure, however, some might even consider neglecting brush cytology during a therapeutic ERCP procedure due to the sensitivity problem. During any therapeutic stenting procedure brush cytology can be performed with no reasonable extra effort, carries a very low complication risk [6], and a positive cytology result spares the patient from further demanding diagnostic procedures such as EUS/FNA or intraductal biopsies. Although it is a highly controversial issue in the EUS era, even diagnostic ERCP-based sampling might still have a role in very carefully selected cases in non-mass forming strictures with clear imaging proof of ductal dilatation, or small lesions with failed FNA due to extensive perilesional inflammation. Intraductal imaging and sampling methods are even expected to help the early diagnosis of pancreatic cancer concomitant with intraductal papillary mucinous neoplasia (IPMN), growing silently in the pancreatic duct with no visible mass lesion available for early image diagnosis [7].

ERCP/brush cytology in jaundiced patients is a valuable technique in pancreatobiliary cancer diagnosis principally in the neoadjuvant and palliative setting, and as an adjunct procedure in indeterminate biliary strictures, but efforts should be made to increase the sensitivity of the procedure. Some ancillary cytological methods have already been tested in clinical studies which identified additional patients with malignancy over routine cytology without additional false positives. These include mutational analysis (ie, KRAS, p16, p53) [8], immunohistochemistry (ie, mesothelin) [9], DNA ploidity tests [10]. All of these tests have shown promise in accurately identifying malignant pancreatobiliary strictures, but with the exception of fluorescence in situ hybridization (FISH), none of them are used in clinical practice at the present. The detection of polysomy by FISH has been shown to double sensitivity in the most comprehensive study to date [10], more recently the same group developed a set of FISH probes that separate pancreatobiliary malignancy with a sensitivity of 64.7% [11]. The evaluation of brushings by both routine cytology and FISH is now common day practice in some cytopathology laboratories [12]. FISH based cytology and more recently next-generation sequencing, have the potential to approach the desired sensitivity values in the diagnosis of various cancer types including pancreatobiliary malformations, however, the expertise needed and cost of the procedures hampers their widespread use in GI units worldwide [13].

In recent years there has been a dramatic increase in the discovery of microRNAs (miRs) playing important roles in a variety of fundamental cellular processes and helping the early diagnosis of various diseases, mainly cancers [14]. MicroRNAs are disease specific, stable markers that can be detected quickly and reproducibly by PCR based methodology available more widely in regular cytopathology laboratories [15]. Aims of the project were: (1) to prove that microRNAs can be detected and isolated from brush cytology samples, (2) to determine the expression of four tumor-associated microRNAs (miR-16, miR-21, miR-221 and miR-196a) on obtained cytology samples, (3) to give way to novel single or combined molecular markers in order to increase the sensitivity of brush cytology enough to impact clinical decision making.

Patients/materials and methods

Subjects and study design

Clinical data of patients was prospectively collected at the Department of Interventional Gastroenterology, National Institute of Oncology, Budapest, Hungary. A total of 73 samples from Caucasian patients were included into this retrospective study. Tissue samples were collected during n = 57 ERCP procedures. Patients were categorized in the malignant group with (1) histological proof of malignancy (endoscopic or percutaneous biopsy, surgical exploration and sampling, autopsy), (2) clearly malignant clinical course over at least 12 months after sampling (evidence of progression such as large vessel involvement, appearance of malignant lymph nodes or new metastases on imaging), (3) pancreatobiliary tumor-related death during follow-up. Patients grouped under benign stricture had none of the mentioned features and were followed-up for 20 months to exclude progression or malignancy.

After exclusion of duplicates (repetitive sampling for suspected false negatives), parapapillary lesions, metastatic biliary strictures from non-pancreatobiliary primaries, and samples where metastatic origin of a pancreatic mass could not be ruled out, we ended up with n = 35 samples (biliary malignant n = 14, pancreatic malignant n = 12, pancreatobiliary benign n = 9). All malignant biliary brushings were obtained from cholangiocellular carcinoma patients (n = 14). For a summary of patient characteristics we refer to Table 1.

The study protocol was approved by the Institutional Review Board and the Hungarian National Ethical Review Committee ETT-TUKEB (*2372/2012/EKU*). All patients gave written informed consent for sample collection and molecular analysis before the onset of endoscopy. Sample and data management was done anonymously.

Sample procurement

Brush cytology samples were collected during ERCP procedures using a through-the-scope disposable gastrointestinal cytology brush (*Ref# 152, ConMed, Utica, NY, USA*). After preparation and fixation of direct smears for routine cytology analysis during the procedure, the distal brush tip containing the samples was immediately positioned in 1.5 ml empty Eppendorf tubes and stored temporarily in an IsoFreeze-Rack (*KR-20B, Kisker Biotech GmbH & Co., Steinfurt, Germany*) keeping the specimens at −10 °C to −20 °C, and were stored long-term in a −80 °C freezer.

Cytology analysis

The cytology specimens were first evaluated as part of the routine clinical work-up and were later reviewed independently by three pancreatobiliary cytology specialists who were blinded to the clinical records and the results of the genetic tests. The slides were scored on loss of honeycombing, chromatin clumping, increased nuclear/cytoplasmic ratio, nuclear molding, nucleoli, necrosis and inflammation [16]. The smears were graded as negative (A), atypical (B), suspicious (C) and positive (D) for malignancy (Fig. 1) [10]. Throughout the study we consider only category D (high number of malignant cells) as a positive cytology result.

Total RNA isolation

Total RNA including the small RNA fraction was isolated, using a miRNeasy Mini Kit (*Qiagen, Hilden, Germany*) following the protocol provided by the manufacturer. The final volume of elution was 50 μL. Measurements of RNA quality and quantity were performed with Qubit 1.0 fluorometer and Nanodrop (*ThermoFisher Scientific, Waltham, MA, USA*). Each sample was loaded on RNA Nano gel electrophoresis chips and visualized on the Agilent 2100 Bioanalyser (*Agilent Technologies, Santa Clara, CA, USA*) for quality of the small RNA band (from 25 to 200 nt).

Table 1
Clinical characteristics of the patients.

	malignant biliary strictures n = 14, 40%	malignant pancreatic strictures n = 12, 34%	benign pancreatobiliary strictures n = 9, 26%
Diagnosis n (%)	9/14 distal bile duct 5/14 perihilar bile duct	8/12 pancreatic head 4/12 pancreatic body/tail	6/9 chronic pancreatitis 1/9 pseudocyst 1/9 cystadenoma serosum 1/9 cholelithiasis
Staging n (%) [b]	6/14 - Stage II 1/14 - Stage III 7/14 - Stage IV	2/12 - Stage II/A 1/12 - Stage III 9/12 - Stage IV	
Gender (m/f)	10/4	8/4	5/4
Age at sampling (year) [a]	69.1 ± 6.4	63.3 ± 9.2	69.6 ± 10.4
Survival after intervention (month) [a]	11.8 ± 8.7	6.7 ± 9.1	
Bilirubin (μmol/L)[c,a]	198.1 ± 130.7	134.5 ± 131.2	47.7 ± 74.3

[a] mean ± SD.
[b] American Joint Committee on Cancer (AJCC) TNM Staging of Pancreatic Cancer and Bile Duct Tumors (2010).
[c] Pre-ERCP values.

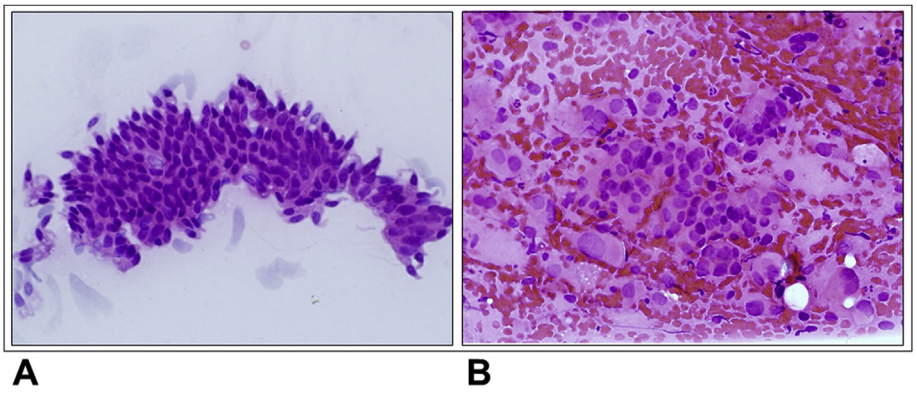

Fig. 1. Representative cytologic specimens from pancreatobiliary brushings: (A) normal ductal epithelial cell sheet - negative (H&E, 200x), (B) tumor cells - positive (H&E, 200x).

MicroRNA quantification by RT-PCR

Quantitative miRNA expression analyses were performed using the Taqman microRNA PCR Assay (*ThermoFisher Scientific, Waltham, MA, USA*). Reverse transcription of 8 ng total RNA (per sample) to cDNA was converted according to the protocol of the Taqman microRNA Reverse Transcription Kit (*ThermoFisher Scientific, Waltham, MA, USA*). Quantitative RT-PCR was performed for hsa-miR-16-5p, hsa-miR-21-5p, hsa-miR-196a and hsa-miR-221-3p. Selection of analyzed microRNAs was based on existing literature suggesting high expression in pancreatobiliary cancer [17–21]. Small nuclear RNA U6 (RNU6B; *Applied Biosystems, Foster City, CA, USA*) was used as internal control. Primer microRNA sequences for the RT-PCR assay are provided in Supplementary Table 1. All real-time reactions were run on 96-well plates in duplicates, using the LightCycler 480 thermocycler (*Life Science Roche*).

Statistical analysis

All C_t (threshold cycle) values were normalized by subtracting RNU6B control values (ΔC_t), and depicted after subtraction from 40. Normalized expression values of 5 samples constantly remained out of range for all microRNA markers studied, therefore these samples were excluded and further statistical calculations were performed on the final set of n = 35 samples. Overexpression of specific microRNAs in malignant samples was calculated using the $\Delta\Delta C_t$ method. We performed statistical analyses using the SPSS software (version 17.0, SPSS Inc., Chicago, USA). ROC analyses were performed with the MedCalc software (version 15.6). Results with $p < 0.05$ were regarded as statistically significant. Quantitative variables were described as mean ± standard deviations.

Results

Malignant disease was present in 26 patients (74%), presenting as biliary and pancreatic strictures in 14 and 12 patients, respectively. Nine patients (26%) had benign pancreatobiliary disease causing stricture formation with prestenotic dilation and clinical symptoms necessitating an intervention. Of the final 35 subjects 11 (31%) had a detectable mass on imaging. Routine cytology resulted in no false positive diagnoses (100% specificity), however, the true diagnosis of malignancy was missed in 46.2% of patients. If we considered suspicious (C) categories positive as well, the sensitivity increased somewhat (73%); however, this was accompanied by a decrease in specificity (89%).

Expression of candidate microRNAs

The isolation of total RNA including microRNAs was successful in each of the analyzed brush cytology specimens, resulting in total RNA quantities of 2.4 ± 1.8 μg per isolation on average with clean qualitative results showing one distinct peak (not depicted). Expression levels of all the analyzed target microRNAs were higher in the brush samples (n = 35) of patients with malignant strictures compared to benign samples after normalization with RNU6B, however, this tendency did not reach statistical significance for miR-21 ($p = 0.1062$) (Supplementary Fig. 1.). No significant expression changes were observed comparing benign pancreatic and biliary samples, therefore we assigned them to a single normal group. Expression (ΔCt values) of miR-16 ($p = 0.0039$), miR-196a

($p = 0.0003$) and miR-221 ($p = 0.0049$) showed a clear statistical significance between malignant and benign pancreatobiliary specimens ($n = 35$), with median ΔCt values differing (ΔΔCt) by 3.5 cycles for miR-16, 4.6 cycles for miR-196a, 2.1 cycles for miR-221, indicating approximately 11.3-fold, 23.6-fold and 4.3-fold increased microRNA levels in the isolates of malignant strictures compared to benign samples (Supplementary Fig. 1.). Subsequent analyses are presented with the best three microRNA markers mainly (miR-16, miR-196a, miR-221). Baseline differences in gender distribution, pre-ERCP bilirubin levels, CA19-9 value and tumor size had no statistically significant effect on microRNA expression levels (not shown).

Subgroup analysis

As the therapeutic indications for ERCP are more common in biliary strictures and published data show differences in diagnostic sensitivity according to location, we next analyzed the data separating biliary and pancreatic malignant samples. Sensitivity of brush cytology was higher in pancreatic strictures (66.7% vs. 42.9%) with a diagnostic accuracy of 81%, although the tendency did not reach statistical significance. Pancreatic brush samples from malignant strictures showed significantly higher expression levels of miR-16 ($p = 0.0098$) and miR-196a ($p = 0.0254$) compared to benign strictures, miR-221 expression was also higher in tumor samples, however, this tendency did not reach statistical significance ($p = 0.0973$) (not shown). In contrast, all target microRNAs were significantly enriched in malignant biliary samples compared to normal pancreatobiliary samples as shown in Fig. 2. MiR-196a proved to be the best marker studied, with significantly higher expression values separating cholangiocellular carcinoma from normal specimens ($p = 2.3 \times 10^{-6}$).

Diagnostic correctness of single and combined microRNA markers

To assess the clinical usefulness of the highly up-regulated single microRNA markers, we next analyzed the data by receiver operating characteristic (ROC) curves. Since the specificity of brush-cytology alone was 100% in our series, we tried to choose optimal cut-off values for the investigated markers with the highest matching sensitivity value without sacrificing the good specificity of cytology. Using a cut-off value $\Delta Ct > -0.985$ for miR-196a malignant and benign pancreatobiliary samples could be separated with a sensitivity of 69.2% and a specificity of 100%, these values could be improved further with the combination of routine cytology and miR-196a expression, resulting in a 84.6% true positivity count, maintaining the PPV at 100%. The combination of several microRNA markers did not improve these statistics (Table 2).

ROC analysis for the subset of pancreatic samples resulted in optimal cut-off values of $\Delta Ct > 7.975$ (miR-16), $\Delta Ct > -1.68$ (miR-196a) and $\Delta Ct > -0.345$ (miR-221), which corresponded to 91.7% sensitivity and 88.9% specificity using miR-16 as the best single marker. Combined analysis (e.g. with cytology or other markers) did not improve the diagnostic performance of miR-16 marker, nor did miR-196a and miR-221 improve the diagnostic precision of routine cytology in pancreatic strictures (Table 3).

When analysing the subset of biliary specimens, the following cut-off values seemed to result in the best diagnostic values: $\Delta Ct > 7.975$ (miR-16), $\Delta Ct > -0.985$ (miR-196a) and $\Delta Ct > -0.345$ (miR-221). MiR-196a as a single marker showed a sensitivity of 85.7% which corresponded to a specificity of 100% (Fig. 3). Combined expression analysis of several markers did not increase diagnostic precision, however, miR-196a and cytology together reached a sensitivity of 92.9% with no false positives (Table 4).

Discussion

In our study we have analyzed microRNA expression as an ancillary test on pancreatobiliary brush cytology specimens obtained during ERCP procedures, and found that expression of miR-16, miR-196a and miR-221 reliably distinguishes malignant from benign pancreatobiliary strictures. Malignancy could be detected combining routine cytology and the miR-196a single marker expression levels with a sensitivity of 84.6% with no false positives.

Routine cytology had an average sensitivity of 53.8% with a specificity of 100%, as expected, in the subgroup analyses sensitivity for pancreatic strictures was higher compared to biliary strictures (66.7% vs. 42.9%) maintaining the perfect specificity (see Tables 2–4). The higher sensitivity value in pancreatic strictures was in line with available literature, which is probably due to the tightness of the strictures (intervention is needed at more advanced stages and the pancreatic duct is narrower to start with) [22]. Our average sensitivity values were higher compared to published data due to the fact that the results were based on a consensus blinded investigation by three expert cytopathologist, and more stringent analysis interestingly resulted in a somewhat higher malignancy detection rate [5]. We must also point out that published sensitivities for biliary sampling tend to be lower in papers where pancreatic head tumors are included, because pancreatic masses compress, but don't necessarily invade the bile duct and therefore a truly negative cytology could erroneously be counted as a false negative one. False negativity could be attributed to well differentiated tumors, and erroneous sampling of the

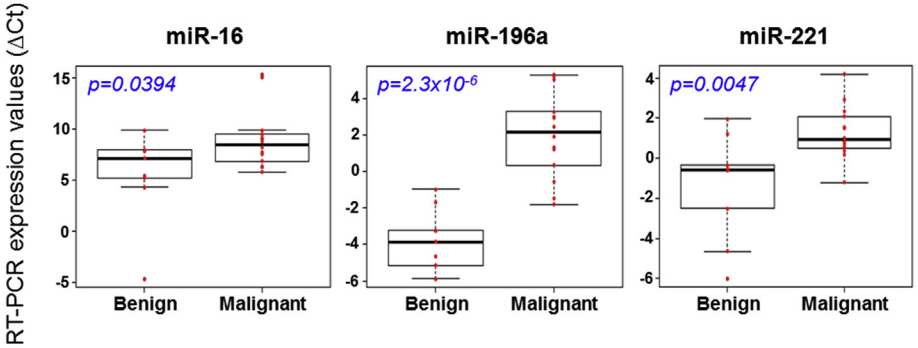

Fig. 2. Box plot presentation of threshold cycle differences between malignant and benign biliary samples for target microRNAs (miR-16, miR-196a, miR-221). The ΔCt values shown are presented after subtraction of the reference gene RNU6B threshold cycle numbers, and final results were depicted after subtraction from 40, therefore a higher number reflects higher value of expression. Results are shown as Box plots. Threshold cycle differences were significant in all cases, with the given p-values. Sample size is $n = 23$.

Table 2
Diagnostic utility of single and combined microRNA markers in the entire group of strictures.

	Cytology	miR-16	miR-196a	miR-221	Combined expression profiles			Cytology + miR-196a	Cytology + miR expression (2 positive)
					1 positive	2 positive	3 positive		
Sensitivity (%)	53.8	73.1	69.2	80.8	96.1	88.5	38.5	84.6	88.5
Specificity (%)	100.0	88.9	100.0	77.8	77.8	88.9	100.0	100.0	88.9
PPV (%)	100.0	95.0	100.0	91.3	92.6	95.8	100.0	100.0	95.8
NPV (%)	42.9	53.3	52.9	58.3	87.5	72.7	36.0	69.2	72.7
Accuracy (%)	65.7	77.1	77.1	80.0	91.4	88.6	54.3	88.6	88.6

PPV = positive predictive value, NPV = negative predictive value, miR = mature microRNA.

Table 3
Diagnostic utility of single and combined microRNA markers in pancreatic strictures.

	Cytology	miR-16	miR-196a	miR-221	Combined expression profiles			Cytology + miR-16	Cytology + miR expression (2 positive)
					1 positive	2 positive	3 positive		
Sensitivity (%)	66.7	91.7	66.7	66.7	91.7	91.7	41.7	91.7	91.7
Specificity (%)	100.0	88.9	88.9	77.8	77.8	88.9	88.9	88.9	88.9
PPV (%)	100.0	91.7	88.9	80.0	84.6	91.7	83.3	91.7	91.7
NPV (%)	69.2	88.9	66.7	63.6	87.5	88.9	53.3	88.9	88.9
Accuracy (%)	81.0	90.5	76.2	71.4	85.7	90.5	61.9	90.5	90.5

PPV = positive predictive value, NPV = negative predictive value, miR = mature microRNA.

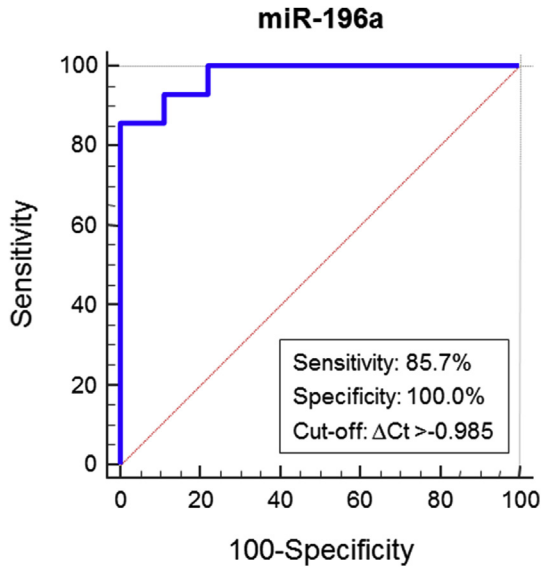

Fig. 3. ROC analysis for miR-196a as a diagnostic biomarker for malignancy in biliary brushings. Sensitivity is plotted versus 100% - specificity.

"normal" tissue adjacent to the tumor, on the other hand previous endoscopic manipulation (infection, mechanical causes) might result in "overstaging" cytology not observed in our set of samples (not shown). Although ERCP/brush cytology of parapapillary masses is important, we handled these tumors separately, as the organ of origin is hard to define, and in some cases even macroscopic pathology examination fails to conclude on the site of origin confusing statistical calculations. In parapapillary cases using endpoints based on brush cytology is not acceptable due to the possibility of false negatives, which would result in misclassification of the patient.

A prospective head-to-head comparison of EUS/FNA and ERCP mediated sampling resulted in comparable sensitivities for biliary masses (79% for both groups), underlining the utility of ERCP/brush cytology in indeterminate biliary strictures, however, the authors conclude to start with EUS first based on the higher incidence of pancreatic masses [23]. In the case of sampling pancreatic masses the superiority of EUS is evident, however, a more reliable comparison of the sampling methods per se would be possible by techniques minimizing technical obstacles, such as repeated sampling: one published sensitivity of EUS/FNA for pancreatic masses after a single needle pass is 17% (with gradual increase to 87% when more than seven passes were performed), which is well below the 66.7% sensitivity of ERCP/brush cytology after one sampling in our cohort [24], and repetitive brushings are known to increase the diagnostic yield of brush cytology as well [25]. Not to mention on-site cytopathology, which is known to improve EUS/FNA accuracy and hence could do the same for the accuracy of the ERCP brushings [26]. An advantage of ERCP guided sampling is the lack of tumor seeding, a known risk described in EUS/FNA patients [7,27].

There is a growing body of literature about the epigenetic molecular markers, microRNAs (miRs), which can be utilized in clinical applications based on the stability and the disease-specific

Table 4
Diagnostic utility of single and combined microRNA markers in biliary strictures.

	Cytology	miR-16	miR-196a	miR-221	Combined expression profiles			Cytology + miR-196a	Cytology + miR expression (2 positive)
					1 positive	2 positive	3 positive		
Sensitivity (%)	42.9	57.1	85.7	92.9	100.0	85.7	50.0	92.9	85.7
Specificity (%)	100.0	88.9	100.0	77.8	77.8	88.9	100.0	100.0	88.9
PPV (%)	100.0	88.9	100.0	86.7	87.5	92.3	100.0	100.0	92.3
NPV (%)	52.9	57.1	81.8	87.5	100.0	80.0	56.3	90.0	80.0
Accuracy (%)	65.2	69.6	91.3	87.0	91.3	87.0	69.6	95.7	87.0

PPV = positive predictive value, NPV = negative predictive value, miR = mature microRNA.

expression of these small RNA molecules. MicroRNAs have been reported to have important functions in the regulation of carcinogenesis and cancer progression as well as homeostasis. Deregulated microRNAs can give information on transcriptional regulation and most importantly may serve as biomarkers for survival and early detection of pancreatobiliary cancers [15]. The aim of the present study was to analyze whether determination of microRNA expression levels in intraductal brush cytology specimens is a viable molecular technique and if the tumor-associated microRNA expression in brush cytology specimens is a valuable tool in the diagnosis of pancreatobiliary cancers.

The isolation of microRNAs was successful from brush cytology specimens, RT-PCR expression analyses of miR-16, miR-196a and miR-221 showed a clear and reproducible statistical significance between malignant and benign pancreatobiliary specimens, indicating approximately 11.3-fold, 23.6-fold and 4.3-fold increased microRNA levels in tumor tissue. Interestingly miR-21, a well-established diagnostic and prognostic marker in other sample sources [28], was not useful as a separation marker on brush cytology isolates in our cohort. Target microRNAs were significantly enriched in the subgroup analysis of malignant biliary specimens compared to normal samples (Fig. 2). MiR-196a proved to be the best marker for pancreatobiliary brushings in general, and even more so for the biliary subgroup, with highly different expression values separating cholangiocellular carcinoma from normal specimens ($p = 2.3 \times 10^{-6}$). The prognostic value of miR-196a expression has not been tested in our study, however, it has been shown recently that high levels of miR-196a as a plasma biomarker is associated with dismal survival in pancreatic adenocarcinoma patients [29]. ROC analyses for the miR-196a marker in combination with routine cytology showed that malignant and benign pancreatobiliary samples could be separated with a sensitivity of 84.6% and a specificity of 100% (Table 2). For the biliary subgroup miR-196a and cytology together reached a diagnostic sensitivity of 92.9% with no false positives (Table 4), the combination of several microRNA markers did not improve these statistics. Feldmann et al. analyzing 16 samples found that routine cytology combined with the detection of HoxB7 and HAAH messenger RNA increased the overall diagnostic sensitivity from 36% to 82% in biliary strictures [30]. In 2012 Nischalke et al. demonstrated that gene expression of a combination of messenger RNA markers (IGF2BP3, HOXB7 and NEK2) from brush cytology specimens is a helpful adjunct to routine cytological readings resulting in sensitivities of 87.5% and specificities of 87.2% in the diagnosis malignant biliary strictures [31]. Compared to messenger RNA (mRNA) markers, microRNA markers have several advantages (1) unlike screening for large numbers of mRNA expression (microarray approach of the past decade), a modest number of microRNAs might be sufficient to differentiate disease from normal (e.g. miR-196a above); and (2) unlike mRNAs, microRNAs in tissues and biofluids remain largely intact and have been proven more stable for detection even in archived paraffin-embedded samples [32].

ERCP procedures are performed in big numbers worldwide for therapeutic interventions mainly in the biliary system, with the possibility of sample acquisition in the same endoscopic session. Besides well-established indications in the neoadjuvant and palliative setting in jaundiced tumor patients, ERCP sampling might still have a role in pancreatic strictures with small (<1 cm) or no detectable mass on imaging due to the shortcomings of EUS/FNA in these settings, especially in high-risk individuals for pancreatic adenocarcinoma. To generalize the use of brush cytology in the diagnostic work-up, supplementary techniques are needed to increase sensitivity to an acceptable level. The unique advantage of the RT-PCR based method to measure microRNAs in cytology brushings presented in our paper over other published techniques is that it is a cheap, fast and widely available tool in all centers to reliably separate tumors from benign samples based on microRNA molecular markers. In our routine, sample processing took 2 h and the estimated cost of molecular analysis was 30 € per patient. We have analyzed microRNA expression on pancreatobiliary brush cytology specimens for the first time, and miR-196a as a single marker showed a sensitivity of 85.7% in biliary strictures, which corresponded to a specificity of 100% (Fig. 3). Shortcomings of our study are the small sample size, single-center retrospective design and the lack of a separate validation group for optimal cut-off values. A follow-up study on a big sample size is currently ongoing in our laboratory. In conclusion, if the good diagnostic performance can be replicated on a large set of samples, microRNAs have the potential of becoming biomarkers increasing the sensitivity of ERCP/brush cytology.

The authors declare no conflict of interest. Parts of this project were highlighted as an oral presentation at the Combined Meeting of the European Pancreatic Club and International Association of Pancreatology (2014, Southampton, UK).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pan.2019.04.001.

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