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ORIGINAL ARTICLE

Comparison of Native Aspirates and Cytological Smears Obtained by EUS-Guided Biopsies for Effective DNA/RNA Marker Testing in Pancreatic Cancer

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Abstract

We compare two types of pancreatic carcinoma samples obtained by EUS-guided fine needle biopsy (EUS-FNB) in terms of the success rates and clinical validity of analysis of two most commonly investigated DNA/RNA pancreatic cancer markers, *KRAS* mutations and miR-21 expression. 118 patients with pancreatic ductal adenocarcinoma underwent EUS-FNB. The collected sample was divided, one part was stored in a stabilizing solution as native aspirate (EUS-FNA) and second part was processed into the cytological smear (EUS-FNC). DNA/RNA extraction was followed by analysis of *KRAS* mutations and miR-21 expression. For both sample types, the yields of DNA/RNA extraction and success rates of *KRAS* mutation and miRNA expression were evaluated. Finally, the resulting *KRAS* mutation frequency and miR-21 prognostic role were compared to literature data from tissue resections. The overall amount of isolated DNA/RNA from EUS-FNC was lower compared to the EUS-FNA, average yield 10 ng vs 147 ng for DNA and average yield 164 vs. 642 ng for RNA, but the success rates for *KRAS* and miR-21 analysis was 100% for both sample types. The *KRAS*-mutant detection frequency in EUS-FNC was 12% higher than in EUS-FNA (90 vs 78%). The prognostic role of miR-21 was confirmed in EUS-FNC ($p = 0.02$), but did not reach statistical significance in EUS-FNA ($p = 0.06$). Although both types of EUS-FNB samples are suitable for DNA/RNA extraction and subsequent DNA mutation and miRNA expression analysis, reliable results with clinical validity were only obtained for EUS-FNC.

Keywords EUS-FNA · Pancreatic cancer · KRAS · miR-21

Introduction

Pancreatic cancer with its most common subtype, the pancreatic ductal adenocarcinoma (PDAC), is one of the most feared cancers because of its dismal survival prognosis of just several months in most cases. Due to the absence of clinical symptoms in its early phase, PDAC is typically diagnosed in advanced inoperable stages, which are characterized by a very rapid progression. Currently, there is a lack of reliable biochemical markers useable in diagnosis and/or management of the disease. The only tumor marker used in clinical practice, the CA19–9 (carbohydrate antigen 19–9), has a limited use in the diagnosis of PDAC with its relatively low sensitivity and specificity (79–81% and 82–90%, respectively) [1].

In the field of molecular markers based on DNA or RNA, a proto-oncogene *KRAS* has been widely investigated in PDAC. Mutation-activated *KRAS* is a well-known driver of initiation and progression of PDAC. Determination of the presence of point mutations in the *KRAS* can mainly be used in a differential diagnosis of focal lesions (chronic pancreatitis vs. carcinoma), as *KRAS* mutation occur in up to 93% of pancreatic cancers [2], while they are virtually absent in the tissue of chronic pancreatitis [3, 4]. *KRAS* mutation status could potentially have a predictive role in a targeted biological therapy,

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but no inhibitors of KRAS signaling pathway have yet been demonstrated to have any effects in PDAC treatment [5]. Therefore, attention is now put on key effectors downstream of KRAS signaling, particularly phosphoinositide 3 - kinase (PI3K) and mitogen - activated protein kinase (MAPK) pathways, through which it would be possible to target the KRAS indirectly [5, 6]. Currently, more than 40 PI3K and more than 20 MAPK inhibitors are included in clinical trials, so it is likely that in the future will the mutation status of the *KRAS* gene have a predictive role [6]. Most recently, we found that a certain type of *KRAS* mutations is an important prognostic marker [7]. It is therefore clear that KRAS is in PDAC key marker with great future potential.

Micro RNAs (miRNAs) present another promising class of biomarkers. An aberrant expression of miRNAs that induce silencing of key signaling pathway genes has been described in a variety of cancers, including PDAC. Increased or decreased levels of these miRNA can then cause incorrect cell signaling, and thus contribute to malignant transformation. In a recent review [8], we have listed miRNAs that are involved in carcinogenesis of pancreatic tissue. Expression of the most prominent, miR-21, is significantly increased in PDAC compared to non-malignant tissue [9–15] and in addition, its high levels correlate with shorter overall survival [9, 11, 13–16]. Hence, miR-21 represents not only a promising diagnostic but also prognostic marker for clinical practice.

Analysis of above mentioned molecular markers, *KRAS* and miRNA, are currently mainly performed on the resected tissue of pancreatic tumors. This, however, is relevant only for a small subgroup of PDAC patients (ca.15%) as the advanced disease typically prevents undergoing of surgical treatment. The endoscopic ultrasound-guided fine-needle biopsy (EUS-FNB) remains the main source of tumor tissue applicable to all patients as other alternative sample processing approaches (such as cell-blocks) inherently suffer from DNA degradation and chemical modification due to the formalin fixation. At the same time with other sample types tumor DNA yield is reduced due to the high content of wildtype DNA coming from lymphocytes. Hence EUS-FNB with subsequent cytological evaluation is currently a standard diagnostic procedure for PDAC [17].

However, use of EUS-FNB samples for molecular analysis is still rare. This is probably due to the low content of representative material with sufficient quality compared to the resection samples. Development of reliable analysis methodology of the molecular markers from EUS-FNB samples is therefore crucial for their introduction to the clinical use.

EUS-FNB samples of pancreatic tissue are processed into smears for cytological diagnosis. In the past, we and others have demonstrated feasibility of the cytological smears (EUS-FNC) for *KRAS* mutation detection [3, 18, 19]. However most pathologists performing *KRAS* testing from EUS-FNB samples prefer native cellular aspirate (EUS-FNA) as the source material [20–23]. Although EUS-FNA allows the extraction of a sufficient amount of high quality DNA/RNA, the sample is not evaluated by a cytologist and accurate representation of the tumor cells is uncertain. Confirmation of the presence of tumor cells is then done indirectly by parallel evaluation of EUS-FNC prepared from a different part of the collected aspirate. It seems, therefore, that a better approach is to perform *KRAS* analysis of cellular material directly from EUS-FNC only on selected tumor cells to prevent contamination by leukocytes, stroma and other non-malignant cells.

The use of EUS-FNB samples for analysis of miRNA expression is also rare. Existing EUS-FNB studies of miRNA mainly utilized the EUS-FNA [24–26] as a source material. Compared to mutations, determining miRNA levels requires more precise information of the amount of tumor cells within the sample (cellularity), as the contaminating elements may exhibit significantly different miRNA expression resulting in false results. Therefore, analysis of EUS-FNC is a better approach for determination of miRNA levels than of EUS-FNA. Surprisingly, miRNA analysis from EUS-FNC in PDAC has not yet been described unlike other cancers [27].

Building on our previous studies of somatic mutations in PDAC cytological smears [3, 18], the aim of this study was to investigate the feasibility of miRNA analysis and to compare the utility of two common EUS-FNB sample types - EUS-FNC and EUS-FNA commonly acquired in clinical practice. Both specimen types were evaluated in terms of the DNA/RNA extraction yields and subsequently, detecting *KRAS* mutations and miR21 expression, by the outcome and clinical validity of the molecular genetic testing.

Materials and Methods

Patients

The study design was reviewed and certified by the Scientific and Ethics boards of the Military University Hospital. All patients admitted into the study have signed an informed consent. The study prospectively recruited a total of 120 patients who were diagnosed with PDAC based on the EUS examination, supplemented by EUS-FNC evaluation. Two patients were subsequently excluded because of tumor duplicity. The characteristics of the final group of 118 patients are listed in Table 1.

EUS-FNB Sampling

EUS was performed using a linear echo endoscope GF 180 UCT (Olympus), and the tumor was collected by a standard 22G FNA needle. The resulting sample types evaluated in this work are shown in Fig. 1.

Table 1 Patient characteristics

Total patients		118 (100%)
Gender	Male	64 (54%)
	Female	54 (46%)
Location of PDAC	Head	81 (69%)
	Body	28 (24%)
	Tail	9 (8%)
Disease stage	II	3 (3%)
	III	54 (46%)
	IV	58 (49%)
	unknown	3 (3%)

In all cases, part of the obtained material (Fig. 1a) was smeared onto a slide and submitted to cytological evaluation for final confirmation of PDAC diagnosis. In order to preserve DNA there was no fixation, cytology slides were air dried and then May-Grünwald-Giemsa staining was used upon examination, areas of tumor cells (at least 80%) were marked by the cytologist for subsequent genetic analysis as shown in Fig. 1b. The remaining fresh aspirate was placed into a stabilizing solution (RNAlater, Ambion ThermoFisher Scientific, Grand Island, New York, USA) at room temperature and the next day put at −20 °C until processing for genetic testing. A total of 118 EUS-FNA and 118 EUS-FNC were processed.

Extraction of Nucleic Acids from Native Aspirates and Cytological Specimens

Material from the cytological slides in the marked areas of tumor cells was dissected and transferred into a tube. The remaining parts from the original native aspirates (Fig. 1a) placed in stabilization solution were first slowly thawed, then centrifuged to create a pellet and, finally, the supernatant stabilizer solution was removed. From both sample types, the total RNA and DNA was isolated using the RecoverAll™ Total Nucleic Acid Isolation Kit (Ambion ThermoFisher), which is primarily designed for the extraction of nucleic acids from formalin fixed paraffin embedded (FFPE) blocks. Nucleic acids were isolated according to the manufacturer's instructions; only the initial step (deparaffinization) was omitted. The concentration of nucleic acid was measured with a fluorimeter (Qubit 2.0, Invitrogen, Carlsbad, California, USA) using a RNA HS Assay Kit with a detection limit of 20 ng/ml and a dsDNA HS Assay kit with a detection limit of 0.5 ng/ml (both from Invitrogen).

Evaluation of KRAS Mutations

Detection of somatic *KRAS* mutations covering hotspot in exon 2 (codons 12 and 13) was performed by PCR followed by denaturing capillary electrophoresis (DCE) on an ABI Prism 3100 genetic analyzer (Applied Biosciences, Foster City, California, USA). The technique is based on a principle of differential denaturation of wild-type and mutant alleles, similar to the high-resolution melting technique. It has previously been applied for *KRAS* testing in PDAC and all experimental conditions have been detailed [3, 18]. The analytical sensitivity of the technique has been experimentally evaluated having a limit of detection at 1% minor allele fraction (MAF) The fragment analysis data evaluation was done using Gene Marker v2.4.2. (Softgenetics LLC, State College, Pennsylvania, USA). Detection limit of the method corresponds to a fluorescence intensity of 700 RFU for the wild-type homoduplex peak (Supplementary materials, Fig. S1). The software enables determination of the fluorescence intensity of the resolved fragment peaks and the fraction of *KRAS* mutated cells in the sample could be calculated from the ratio of peak intensities (see Supplementary materials for more information).

Evaluation of miR-21 Expression

5 µl of total RNA was used for reverse transcription from miRNA to cDNA by the qScript micro RNA cDNA Synthesis kit (Quanta Biosciences, Gaithersburg,

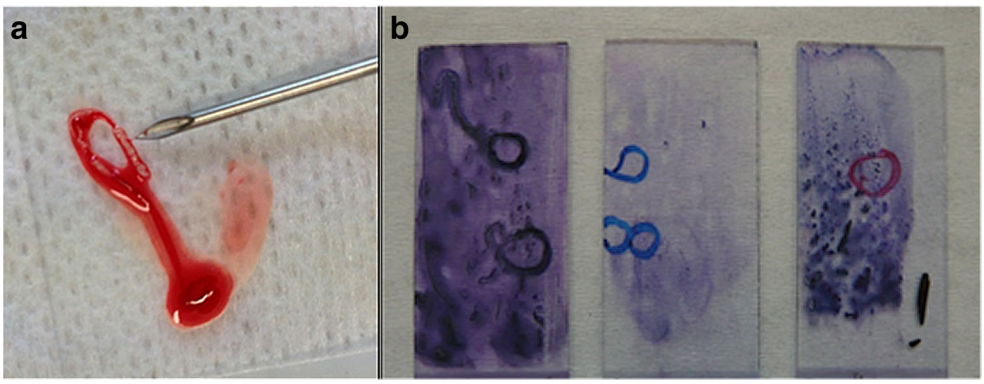

Fig. 1 Sample types acquired by endoscopic ultrasound-guided fine-needle biopsy procedure (EUS-FNB). A native fine-needle aspirate (FNA) (**a**), a fine-needle cytology specimen (**b**)

Maryland, USA) according to the manufacturer's instructions. At first, miRNA was polyadenylated by poly(A) polymerase, then the adenylated miRNA was transcribed to cDNA by reverse transcriptase and oligo dT primer with adaptor sequence. The adaptor sequence allowed for hybridization of a universal primer during the subsequent Real-Time (qRT) PCR.

Amplification and quantification of miR-21 expression by qRT-PCR was performed on a Stratagene Mx3000P (Agilent Technologies, Santa Clara, California, USA) real-time cycler using the PerfeCta micro RNA Assay kit (Quanta Biosciences), PerfeCta Sybr green SuperMix (Quanta Biosciences), a universal primer and a miRNA-specific primer (Quanta Biosciences). Amplification of each sample was done on 2 parallels using the following cycling conditions: initial denaturation at 95 °C /15 min followed by 40 cycles consisting of 94 °C/15 s, 60 °C/30 s and 70 °C/30 s and a final extension at 72°/10 min. Specificity of the PCR products was confirmed by a melting curve analysis of PCR products.

qRT-PCR data was analyzed by MxPro software (Agilent Technologies) with automated baseline setting and 0.4 threshold value. Calculation of relative miR-21 expression was done using an average of Ct values from both parallels. Relative expression of miR-21 was calculated as $2^{-\Delta Ct}$ [28], using RNU6B as a reference gene ($\Delta Ct = Ct_{miR\text{-}21} - Ct_{RNU6B}$). Only results with $Ct \leq 35$ were considered valid.

Statistical Analyses

Fisher's exact test was applied to compare percentages of *KRAS* mutant cells in EUS-FNA and EUS-FNC. $P < 0.05$ was considered statistically significant.

The prognostic value of miR-21 was tested by means of overall survival analysis using Kaplan-Meier method. At first, a median of relative miR-21 expression from all 118 PDAC samples was determined. Then, any value greater than the median was considered as "high expression", while values lower than the median were assigned as "low expression". Overall survival was calculated from the time of diagnosis. In order to evaluate a homogeneous group, patients with unconfirmed survival status ($n = 7$), those who died due to causes unrelated to the PDAC diagnosis ($n = 7$) and those who underwent surgical treatment ($n = 13$) were excluded from survival analysis. In the resulting group of 91 patients, the overall survival for high and low values of miR-21 expression were evaluated by Kaplan-Meier analysis using the Medcalc statistical software (Medcalc, Oostende, Belgium). Survival curves were compared using a log-rank test at 5% statistical significance ($P < 0.05$).

Results

DNA/RNA Yields and Success Rates for KRAS and miR-21 Analysis

Due to the smaller amounts of input material in EUS-FNC, the DNA/RNA extraction yields were approximately an order of magnitude below the yields from EUS-FNA. For DNA, the average yield was 10 ng per specimen (ranging from 1.5 to 58.5 ng) with 19% (22/118) below the fluorimeter detection limit. For RNA, 81% (21/26) of specimens were below the detection limit with the rest containing on average 164 ng of RNA (ranging from 92 to 294 ng).

In EUS-FNA, the yields reflected input amounts of collected pancreatic tissue that was processed as a whole and therefore exhibited considerable variability. For DNA, the average yield was 147 ng (ranging from 6.5 to 1930 ng) with 5% (6/118) of samples below the fluorimeter detection threshold. For RNA, the average was 642 ng (ranging from 138 to 45,000 ng) with 15% (18/118) of samples below the detection limit.

Despite the generally low DNA/RNA yields from both types of EUS-FNB collected specimens, there was a 100% success rate for the detection of *KRAS* mutations and 100% success rate for the analysis of miR-21 expression. At *KRAS* assay, none of the samples produced fluorescence intensity of the wild-type homoduplex below 1200 RFU. For miR-21 expression analysis, the Ct values were in the range of 20.37–33.47 for EUS-FNC and in the range of 17.9–29.43 for EUS-FNA.

KRAS Mutation Detection Rates and the Mutant Cell Fraction

The rate of *KRAS*-mutant positives in EUS-FNC was 90% (106/118), while only 78% (92/118) in EUS-FNA. *KRAS* mutant detection results from both specimen types are compared in Table 2.

Next, the *KRAS*-mutant cell fraction in samples was evaluated (see Supplementary materials for details). The results are illustrated in Fig. 2. As expected, EUS-FNC, where the DNA was extracted only from an area of predominantly tumor

Table 2 Comparison of *KRAS* status determined from both types of EUS-FNB samples

KRAS status determined from native aspirate / cytological smear	% (n/ total)
mutant / mutant	78% (92/118)
wild type / wild type	10% (12/118)
mutant / wild type	0% (0/118)
wild type / mutant	12% (14/118)

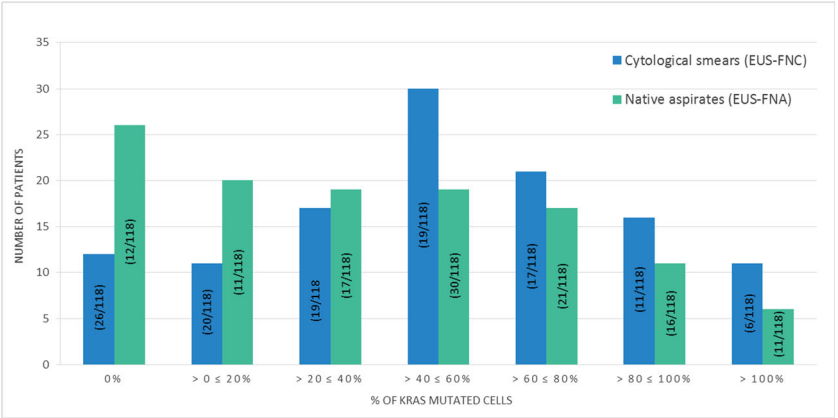

Fig. 2 *KRAS*-mutant cell fraction in EUS-FNC vs EUS-FNA samples

cells marked by an experienced cytologist, contained more *KRAS* mutant cells than in EUS-FNA. The average fraction of *KRAS*-mutant cells in EUS-FNC was 56% compared to 31% in EUS-FNA. Two thirds of EUS-FNC (78/118) contained more than 40% of *KRAS* mutant cells, the same percentage of *KRAS* mutant cells was present only in less than half (53/118) of EUS-FNA ($p = 0.0016$, Fisher's exact test).

miR-21 Expression Analysis and Confirmation of its Prognostic Role

The validity of results obtained from miR-21 expression experiments was assessed by confirming its negative prognostic role as reported by multiple studies done on resected PDAC tissue. The results of Kaplan-Meier survival analysis are shown in Fig. 3a and b. In EUS-FNC a total of 48 patients displayed tumors with low levels of miR-21 expression with a median of overall survival of 200 days, while 43 patients had tumors with high miR-21 expression and a median of overall survival of 128 days. The Kaplan-Meier analysis showed a statistically significant difference confirming the negative prognostic value of miR-21 expression ($P = 0.02$), Fig. 3a. In EUS-FNA, there were 43 patients with tumors showing low miR-21 expression with a median of survival of 208 days and 48 patients with tumors showing high miR-21 expression with a median of survival of 117 days. Despite the difference of almost 100 days between the groups, this result is not statistically significant ($P = 0.06$), see Fig. 3b.

Discussion

Molecular analysis performed from pancreatic tissue collected by EUS-FNB is not routinely used, particularly due to the minute amount of material obtained. The approach, if adopted, would open possibility for molecular testing of virtually all PDAC patients, especially those in inoperable status. Compared to resected tissue, analysis of molecular markers from biopsy specimens is considerably more challenging. The representative material in the sample is reduced due to contribution from blood and a desmoplastic character of the PDAC (a small fraction of malignant cells in an excess of tumor stroma) composing up to 90% of the sample volume [29]. As a result, most research studies evaluating molecular markers in PDAC use resected tissue with very limited relevance for clinical practice.

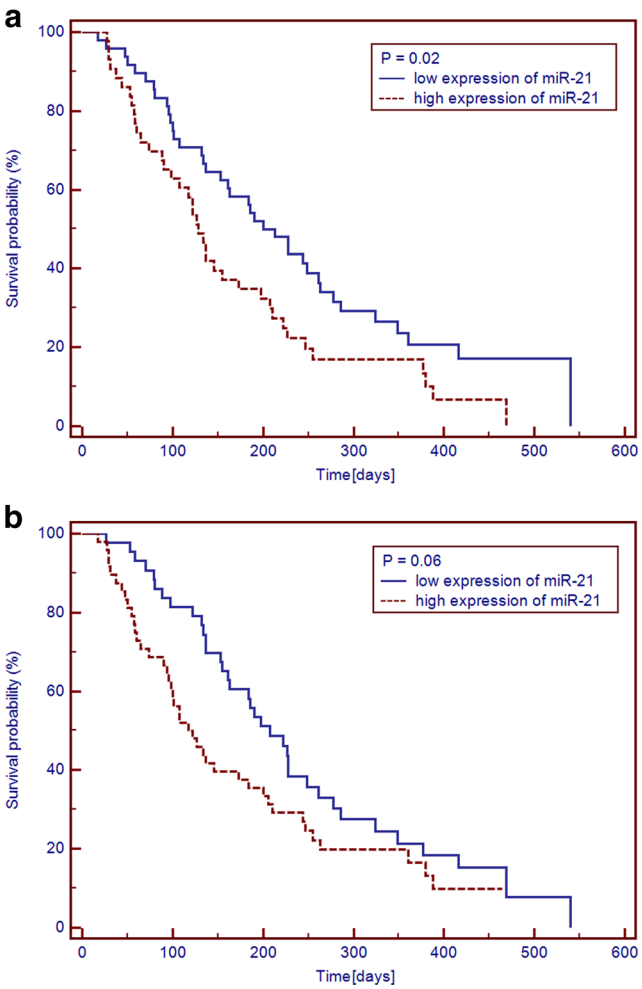

Fig. 3 Overall survival of patients according to miR-21 expression. Comparison of EUS-FNC (**a**) and EUS-FNA (**b**) samples

Both, collection of cells by dissection from EUS-FNC and the direct processing of EUS-FNA, have potential limitations. In the case of EUS-FNC, the fixation process and limited cell amount in sample could decreased quality and quantity of extracted DNA/RNA, while in case of EUS-FNA the indeterminate fraction and representation of tumor cells may lead to a false interpretation of results.

Yet, our results indicate that despite the low amounts of DNA/RNA extracted from EUS-FNC, the ultimate success and reliability was comparable to the EUS-FNA for both DNA and miRNA tests (100% in both cases). Similarly to other cancers preparation of cytological sample has no noticeable impact on the outcome of DNA or miRNA analyses [30]. This is most likely due to very short DNA/miRNAs segments studied (about 100 bp in the case of DNA and 20 bp in case of miRNA), that do not undergo degradation during sample fixation.

Our overall rates of mutant *KRAS* in EUS-FNC samples in PDAC patients was similar to result of Biankin et al. obtained for homogeneous resected tumor mass [2]. In contrast rate of only 78% was obtained for mutant *KRAS* in EUS-FNA samples from the same patients. The 12% of presumably falsely negative EUS-FNA samples were most likely a result of absence of cancer cells in the sample. In addition, the average fraction of tumor cells (tumor cellularity), derived from the *KRAS* mutated DNA fraction within the sample, was close to double in EUS-FNC than EUS-FNA (56 vs 31%). Hence, the knowledge of the tumor cellularity is key for correct data interpretation.

Apparently, EUS-FNC samples seem also more suitable for miRNA analysis. This conclusion can be drawn from the results of the miR-21 expression, where its negative prognostic role known from previous studies [9, 11, 13–16] was confirmed only for EUS-FNC samples ($p = 0.02$). In the EUS - FNA samples the presence of non-tumorous mass obviously influenced the final expression value.

In summary, EUS-FNC samples are a perspective source for molecular markers analysis. Although exhibits much lower yields of nucleic acids, clinical validity of the data obtained is significantly higher.

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