Liver, Pancreas and Biliary Tract

PDX-1 mRNA expression in endoscopic ultrasound-guided fine needle cytoaspirate: Perspectives in the diagnosis of pancreatic cancer

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\textbf{A B S T R A C T}

\textbf{Background and aims:} Endoscopic ultrasound-guided fine needle aspiration is routinely used in the diagnostic work up of pancreatic cancer but has a low sensitivity. Studies showed that Pancreatic Duodenal Homeobox-1 (PDX-1) is expressed in pancreatic cancer, which is associated with a worse prognosis. We aimed to verify whether the assessment of PDX-1 in endoscopic ultrasound-guided fine needle aspiration samples may be helpful for the diagnosis of pancreatic cancer.

\textbf{Methods:} mRNA of 54 pancreatic cancer and 25 cystic lesions was extracted. PDX-1 expression was assessed by Real-Time PCR.

\textbf{Results:} In all but two patients with pancreatic cancer, PDX-1 was expressed and was found positive in 7 patients with pancreatic cancer in which cytology was negative. The positivity was associated with a probability of 0.98 (95\% CI 0.90–1.00) of having cancer and the negativity with one of 0.08 (95\% CI 0.01–0.27). The probability of cancer rose to 1.00 (95\% CI 0.97–1.00) for patients positive to both PDX-1 and cytology and fell to 0.0 (95\% CI 0.00–0.15) in patients negative for both.

\textbf{Conclusions:} PDX-1mRNA is detectable in samples of pancreatic cancer. Its quantification may be helpful to improve the diagnosis of pancreatic cancer.

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1. Introduction

Endoscopic ultrasound (EUS) is routinely used in the diagnostic work up of pancreatic cancer (PC) \cite{1,2}. In particular, EUS-guided fine needle aspiration (EUS-FNA) has proven to be a major advance in light of its ability to obtain PC samples for cytological diagnosis \cite{3,4}. Although EUS-FNA is considered the most reliable and safe procedure for tissue sampling \cite{4}, the probability to achieve a definitive diagnosis is limited by the fact that conventional cytology has high specificity but poor sensitivity \cite{4,5}. A major issue that contributes to limit the diagnostic efficacy of EUS-FNA is the sampling error. The rapid on site evaluation (ROSE) of the adequacy of the sample by a cytopathologist is estimated to increase the sensibility of the procedure by 10–15\% \cite{4}. Unfortunately, the availability of ROSE is still limited in endoscopy units. Efforts are thus being made to improve the diagnostic accuracy of EUS-FNA. To this regard, the application of molecular biology to analyse EUS-FNA samples is thought to be the most promising approach \cite{5,6}.

Pancreatic Duodenal Homeobox 1 (PDX-1) is a transcription factor that is required for the embryologic development of the endocrine pancreas \cite{7,8}. In the adult pancreas, its activation plays an important role for cell proliferation and for insulin synthesis \cite{9}.

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Interestingly, PDX-1 may also play a role in the development of PC [10]: studies have shown that 41–90% of resected PCs are positive for PDX-1 at immunohistochemistry [11–13] and that PDX-1 expression correlates with a poorer prognosis [11,12].

Therefore, we wondered whether the assessment of PDX-1 in EUS-FNA samples may be helpful for the diagnosis of PC. We designed a proof-of-concept study that aimed to answer the following questions: (a) is it possible to assess and quantify PDX-1 mRNA expression in EUS-FNA samples of pancreatic lesions? (b) Does PDX-1 mRNA expression differ in PC vs. non-malignant lesions? (c) Are PDX-1 mRNA levels associated with PC?

2. Materials and methods

2.1. Patients

All the patients referred for pancreatic EUS were enrolled prospectively at four Italian tertiary endoscopic centres over a 6-month period (December 2011–May 2012).

Patients presenting either with a solid or a cystic mass underwent EUS-FNA according to established standards of practice. A small aliquot of the aspirate was devoted to PDX-1 dedicated vial. Reasons for exclusion were: (a) cases in which the endosonographers judged that all the aspirated material was needed for routine cytology, and (b) patients unwilling to consent to our study. All patients received informative material about the study and expressed their will to participate by a written consent and the study was approved by local ethical committees.

2.2. EUS technique

We used radial (GFU140, Olympus Japan) and linear echoendoscopes (GF-UC140P and GF-UCT140, Olympus Japan) in combination with the ultrasonic processor Aloka Alfa-10 (Aloka, Japan).

The EUS examinations were carried out under conscious sedation using meperidine and midazolam with patients lying on the left lateral decubitus.

All the EUS examinations were performed by experienced endosonographers. One to 4 needle passes, by using 22 or 25 gauge needles, were undertaken to obtain diagnostic material for routine cytology (Table 1). As ROSE was not available at any of the participating centre, the adequacy of aspirated material was evaluated by visual inspection.

Gold standard for diagnosis was represented either by surgical specimen or by long-term follow (at least 12 months).

2.3. Tissue collection and RNA extraction

After having considered adequate the sample dedicated to routine cytology, three to five drops of the remaining aspirate were placed in vials containing 1 ml RNAlater® (Ambion, Monza, Italy) and maintained at +4°C for 24 h. Samples were then stored in a coded fashion at −80°C.

Total RNA was extracted using the TRizol® reagent (Invitrogen, Milan, Italy) then converted to cDNA by employing the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Monza, Italy). 50 ng of cDNA was amplified by Real-Time PCR, by using the SYBR® Green PCR Master Mix (Applied Biosystems, Monza, Italy).

2.4. PDX-1 mRNA expression in EUS-FNA samples

PDX-1 mRNA expression was assessed by Real-Time PCR. Primers were designed with Oligo 6 software using reference mRNA sequences accessed through Gene Bank; specificity of primers was confirmed by BLAST analysis. Primer sequences are listed in

| Table 1 | Clinical features and endoscopic ultrasonography-fine needle aspiration technical aspects of the cases included in the study. Age and diameters are expressed as mean ± standard deviation. N/A: not applicable. |
|---|---|---|
| | Pancreatic cancer (n = 54) | PNML (n = 27) |
| Gender | | |
| Males | 37 (68%) | 14 (51%) |
| Females | 17 (32%) | 13 (49%) |
| Mean age (years) | 67 ± 9 | 63 ± 15 |
| Localization | | |
| Head | 33 (61%) | 14 (51%) |
| Body | 19 (35%) | 12 (44%) |
| Tail | 2 (4%) | 1 (5%) |
| Mean diameters (mm) | | |
| D1 | 35 ± 11 | 34 ± 12 |
| D2 | 32 ± 10 | 28 ± 13 |
| Cancer stage | | |
| 1 | 8 (15%) | |
| 2A | 9 (17%) | |
| 2B | 11 (20%) | |
| 3 | 10 (19%) | |
| 4 | 15 (29%) | |
| PNML type | N/A | |
| IPMN branch duct | 15 (56%) | |
| IPMN main duct | 2 (7%) | |
| Mucinous cystaden. | 2 (7%) | |
| Serous cystaden. | 5 (19%) | |
| Pseudocyst | 1 (4%) | |
| Chronic pancreatitis | 2 (7%) | |
| Needle size (mm) | | |
| 19 | 1 (2%) | 0 |
| 22 | 2 (7%) | 0 |
| 25 | 11 (20%) | 8 (30%) |
| Needle passes | | |
| 1 | 6 (11%) | 17 (63%) |
| 2 | 35 (65%) | 10 (27%) |
| 3 | 12 (22%) | 0 |
| 4 | 1 (2%) | 0 |

Supplementary Table 1. Real-Time PCR was performed using the Rotor–Gene 6000 (Corbett Research) with SYBR Green fluorophore. A melt curve analysis was performed following every run to ensure a single amplified product for every reaction. Reactions were carried out in at least duplicate for every sample. Duplicate negative controls (no template cDNA) were also run to assess specificity and indicate potential contamination. 18S rRNA was used as a reference gene for normalization.

2.5. Fluorescent In Situ Hybridization

Fluorescent In Situ Hybridization (FISH) was performed according to the instructions of the vendor (Creative Biolabs). Briefly, formalin-fixed, paraffin-embedded tissues were dewaxed, treated with Protein K solution, washed in PBS, denatured at 75 ± 2°C for 5 min. Slides were then dehydrated in ethanol and then air dried. The FISH probe was denatured at 75 ± 2°C for 5 min and then we proceeded with the hybridization of the probe to the specimen on the slide, a coverslip was applied, sealed with rubber cement and proceeded with the incubation at 37–40°C overnight. The slide was then washed with PBS, dehydrated and air dried. Finally DAPI counterstain was applied and a coverslip to hybridization location, the slide was examined under a fluorescence microscope with proper filter set. The human PDX-1-TRITC probe sequence was: GGGAGGTAGAAGGGAGGCGAGGAAAGAAAGAGAGAGTCAGAGAAAGGGAGGAGGAAAGGGGTTGTTAGGTGTTTCGTTTCTCGTTTCTCGTTTCTCGTT.
2.6. Cell lines

The PC cell lines Su86.86, BxPC-3, Panc-1, and MIA-PaCa-2 were a kind gift by Prof. Andrea Galli, (Gastroenterology Unit, Department of Clinical Pathophysiology University of Florence, Italy). The neuroendocrine cell lines CM, BON-1, QGP-1 were a kind gift by Dr. Gabriele Capurso (Digestive and Liver Disease Unit, Faculty of Medicine and Psychology, Sapienza University of Rome at S. Andrea Hospital, Rome, Italy). Cells, were seeded on 6-well plates and maintained in DMEM 4.5 g/L d-glucose (Life technologies) supplemented with 10% foetal bovine serum (FBS), 1% penicillin 10,000 U/ml, 10 mg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO₂.

2.7. Statistical analysis

PDX-1 mRNA levels are reported as relative expression as compared to MIA-PaCa-2. PDX-1 was considered expressed, i.e. positive, for values ≥0.4. Exact logistic regression was used to evaluate the separate and joint association of PDX-1 and cytology with pancreatic cancer [1,2,14,15]. The outcome variable was pancreatic cancer (0 = cyst; 1 = cancer) and the predictors were positive PDX-1 (0 = PDX-1 < 0.4; 1 = PDX-1 ≥ 0.4) and positive cytology (0 = no; 1 = yes). Spearman’s rho was used to evaluate the association between PDX-1, diameters (D1, D2), tumour stage (T), nodes (N) and metastasis (M). Statistical analysis was performed using Stata 13 (Stata Corp, College Station, TX, US) and LogXact 10 (Cytel Inc., Cambridge, MA, US).

3. Results

During the study period 516 patients underwent EUS for pancreatic indications at the 4 participating endoscopic centres. Reasons for exclusion from the study were: EUS-FNA not performed (n = 357), material deemed not suitable for study analysis by the operators (n = 41), patients unwilling to consent (n = 22), therapeutic procedures (n = 15). Our study population was finally constituted by 54 patients affected from PC and 27 patients affected from pancreatic non-malignant lesions (PNML, Table 1).

3.1. PDX-1 mRNA is detectable and quantifiable in EUS-FNA aspirates from PC

Before starting the present study, we performed a pilot study to determine the feasibility and reliability of the PDX-1 assessment.
We compared PDX-1 mRNA expression in an EUS-FNA sample collected from a patient with PC with one from a patient with a PNML (serous cystoadenoma) and with a sample from a non-malignant lymph node. The PC, but not the cystic lesion and the lymph node, turned out positive for PDX-1 mRNA (Supplementary Table 2). Peripheral blood was used as negative control.

To confirm that PDX-1 mRNA detected in EUS-FNA samples is expressed by PC cells, we investigated PDX-1 mRNA distribution within the aspirated cell population by FISH. In the normal pancreas, PDX-1 mRNA was expressed by CK-19 mRNA-negative islet cells [16]. In contrast, PDX-1 co-localized with CK-19 mRNA in EUS-FNA cytoaspirates (Fig. 1). These data thus indicate that PDX-1 mRNA is expressed by PC cells aspirated in the course of EUS-FNA.

To establish a reproducible protocol to quantify PDX-1 mRNA in EUS-FNA aspirates, we assessed PDX-1 mRNA in six different PC and PNET cell lines. The MIA PaCa-2 cell line showed a steady expression of PDX-1 mRNA over several passages in culture (Fig. 2). PDX-1 mRNA expression in MIA PaCa-2 cells was thus employed as a standard to which to compare the expression observed in EUS-FNA aspirates.

3.2. PDX-1 mRNA is expressed in EUS-FNA aspirates of PC but not in those of PNML

Routine cytology was diagnostic in 49 of 56 cases of PC. All cases of PC but 2 were positive for PDX-1 mRNA. 7 cases of PC were negative at cytology and 54 were positive for PDX-1 mRNA (Table 2).

PDX-1 mRNA was negative in 24 of 27 cases of PNML. Of the 3 positive cases, the first was a main duct IPMN that was surgically resected with post-surgical diagnosis of cancer; the second was a mucinous cystoadenoma diagnosed as cancer after cytological analysis of the EUS-FNA aspirate; the third was a main duct IPMN and the patient did not undergo surgery for the very advanced age (86 year old) and the presence of co-morbidities. 3 cases out of 5 PNET resulted positive to PDX-1 mRNA expression (Table 2).

3.3. Combining cytology and PDX-1 offers a better estimate of the probability of PC

24 PNML and 2 PC were PDX-1–negative while 54 PC and 1 PNML were PDX-1–positive. PDX-1 positivity was associated with a probability of 0.98 (exact 95% CI 0.91–1.00) of having cancer and PDX-1 negativity with one of 0.08 (exact 95% CI 0.01–0.25) (Table 3).

25 PNML and 7 PC were negative at cytology while 49 PC and no PNML were positive at cytology. A positive cytology was associated with a probability of 1.00 (exact 95% CI 0.93–1.00) of cancer and negative cytology with one of 0.22 (exact 95% CI 0.10–0.39) (Table 3).

Combining PDX-1 and cytology leads to a better estimate of the probability of cancer. The probability of cancer rose in fact to 1.00 (95% CI 0.97–1.00) for patients positive to both PDX-1 and cytology and fell to 0.00 (95% CI 0.00–0.14) in patients negative to both PDX-1 and cytology. True positive rate was 0.96 (exact CI: 0.88–1.00); true negative rate was 0.96 (exact CI: 0.80–1.00).

Among patients with cancer (n = 56), there was no association between PDX-1 and D1, D2, T, N and M (Fig. 3) (p < 0.05 for all, Spearman’s rho).

4. Discussion

The current study shows that: (a) the determination of PDX-1 mRNA levels in EUS-FNA samples of pancreatic lesions is an effective procedure; (b) PDX-1 mRNA is expressed in pancreatic malignancy but not in non-malignant cystic lesions; (c) increased PDX-1 mRNA levels identify pancreatic cancer with high accuracy; (d) PDX-1 mRNA expression is not associated with cancer size and stage.
PC is the fourth cause of death for malignancy in Western countries [1]. PC is a very aggressive disease: less than 20% of patients present with localized, potentially curable tumours: the overall 5-year survival rate among patients with PC is less than <5% [1].

EUS-FNA is strongly recommended by the National Comprehensive Network guidelines as a safe and effective method for the diagnosis of PC [2,17–19]. Although its specificity is remarkably high (nearly 100%), sensitivity ranges from 60 to 90% [3–5,20]. There are several factors known to affect EUS-FNA sensitivity, mainly related to endosonographers’ experience [4,21–26] and availability of ROSE. The latter, when performed by an expert cytopathologist, increases the sensitivity of the procedure by 10–15% [4,27]. However, ROSE is available in a limited number of EUS units. As a result, about 20% of patients undergoing EUS-FNA remain with an undefined diagnosis [17–19].

Over the past 15 years, there have been major efforts to improve the diagnostic accuracy of EUS-FNA [3]. In particular, attention has been given to the identification of biological markers of PC by applying molecular diagnostic techniques in EUS-FNA samples [3,28]. Some approaches aim to identify genetic abnormalities. Among those, the most promising one is the definition of chromosomal abnormalities by FISH: combination of cytology and FISH increases the sensitivity of EUS-FNA by 11%, with a negligible reduction in specificity [5]. Similarly, the definition of mutated K-ras, p53 and p16 or loss of heterozygosity of DPC4/SMAD4 are all putative methods to increase the diagnostic accuracy of EUS-FNA [28–31]. Other approaches consider RNA expression profiles. Expression of S100A6 mRNA in EUS-FNA samples identifies PC with high accuracy [19]. EUS-FNA samples can be employed to identify a specific molecular profile of PC by determining micro-RNA expression [6].

The current proof-of-concept study suggests that the determination of PDX-1 mRNA in EUS-FNA samples may be helpful in identifying PC. First, we showed that PDX-1 mRNA can be determined in EUS-FNA samples (Table 3). As compared to similar methods for RNA collection in EUS-FNA samples [19], the method does not interfere with daily routine, since no additional needle passes are needed. Since the method is based on Real-Time PCR amplification, a small amount of the sample is sufficient for PDX-1 mRNA detection. To confirm that the transcripts detected by Real-Time PCR are expressed in malignant cells of PC, we performed FISH to localize PDX-1 mRNA in EUS-FNA samples. As shown in Fig. 1, PDX-1 mRNA co-localized with CK-19 mRNA in PC cells. This confirms that Real-Time PCR detects differences in PDX-1 mRNA expression in pancreatic cancer cells. Those findings are in accordance with previous data showing that PDX-1 is found positive by immunohistochemistry in PC but not in normal pancreatic tissue [12]. The origin of PDX-1 mRNA from PC cells is also confirmed by the fact that it turned out negative in EUS-FNA samples from non-malignant pancreatic or non-pancreatic lesions (Table 3).

Next, a PC cell line (MIA PaCa-2) with a steady PDX-1 mRNA expression over different passages was identified, which was central to establish a standard value (Fig. 2). As shown in previous studies [19], Real-Time PCR is able to provide a quantitative value of mRNA levels in different samples when the samples are tested simultaneously. Having available a standard value, in contrast, allows to test the samples at different time points. The current protocol, therefore, was effective in providing not only a qualitative, but also a quantitative, repeatable measure of PDX-1 mRNA expression (Table 2 and Supplementary Table 1). Such a feature is especially important since it makes the protocol suitable not only for research purposes but also for possible clinical use.

When PDX-1 mRNA expression was evaluated in 56 consecutive cases of PC, it was found to be positive in all but 2 cases. Importantly, PDX-1 mRNA was positive in 7 cases for which EUS-FNA (with routine cytology) failed to provide a definitive diagnosis of
cancer (Table 2). The three PNML that expressed PDX-1 mRNA were those which were then found to have features of developing PC (Table 2). The expression of PDX-1 in most of our PC cases is in contrast with earlier studies where PDX-1 was found positive, by immunohistochemistry, in 41% of PC who underwent resection [12]. Our data are, however, in agreement with more recent studies that 87.5% of PC cases express PDX-1 [13].

As the diagnostic performance of PDX-1 mRNA is concerned, we found that PDX-1 mRNA expression is associated with PC and that combining PDX-1 with cytology increases the probability of identifying and excluding PC (Table 3). The test seems thus as effective as other molecular tests for the diagnosis of PC [5,19]. Interestingly, PDX-1 mRNA levels were not associated with tumour dimensions and TNM grading (Fig 3). Previous studies have shown that PDX-1 expression in PC is associated with cancer aggressiveness, a finding explained with the fact that it indicates cell dedifferentiation [11,12]. In these studies, lymph node metastases, TNM grading, pathological grading, tumour cell proliferation and worse prognosis were associated with PDX-1 expression levels [11,12]. We believe that two reasons explain our different findings. First, our study was performed in patients with advanced PC (Table 1) as they are those most commonly referred for EUS-FNA. Other studies were performed in patients subjected to surgical resection in earlier cancer stages [11,12]. Second, PDX-1 expression was evaluated by immunohistochemistry [11,12] whereas we used Real-Time PCR, more sensitive to detect molecular expression.

The lack of an association with cancer staging (Fig. 3), as well as with the number of needle passes and size (data not shown), further reinforces the concept that PDX-1 mRNA levels in EUS-FNA samples are predictive of the malignant nature of the lesion. Measurement of PDX-1 mRNA may be useful in clinical practice as it helps to reach the diagnosis when EUS-FNA cytology is inconclusive. Such patients often undergo a second procedure, with a significant increase in risks for patients and costs for healthcare providers [20,32].

Further, specifically designed studies are needed to confirm the diagnostic performance of PDX-1 mRNA detection to discriminate malignant vs. non-malignant solid tumours, since the number of cases of solid lesions in the current series is limited. Further studies are also needed to verify any possible role of such a test in neuroendocrine tumours. However, the current proof-of-concept study was designed to verify if PDX-1 mRNA detection in EUS-FNA samples is effective in identifying PC, an hypothesis that was confirmed by the high predictability of PC with a positive test.

In summary, this study proposes a method to increase the accuracy of EUS-FNA to diagnose or exclude PC. Such method is inexpensive, rapid, reproducible, independent from the amount of sampled tissue (or cystic vs. solid) and, most importantly, does not interfere with routine clinical practice of EUS-FNA. Quantification of PDX-1 mRNA may be helpful to improve the diagnostic performance of EUS-FNA for the diagnosis of PC.

Conflict of interest
None declared.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jdd.2014.10.010.

References