Successful application of endoscopic ultrasound-guided fine needle biopsy to establish pancreatic patient-derived tumor xenografts: a pilot study

Authors
Els Hermans1,*, Schalk W. Van der Merwe2,3,*, Jeroen Depreeuw1,4,5, Jeroen Dekervel2,3, Enrico Radaelli6, Tania Roskams1, Jos van Pelt Joś3, Baki Topal4, Chris Verslype2,4, Hans Prenen1, Werner Van Steenbergen2, Frederik Nevens2, Diether Lambrechts4,5, Frédéric Amant1,9

Institutions
Institutions are listed at end of article.

Background and study aim: Typically, pancreatic patient-derived tumor xenografts (PDXs) are established by transplanting large tumor biopsies obtained through invasive surgery approaches into immunocompromised mice. We aimed to develop pancreatic PDXs by transplanting tumor tissue acquired by endoscopic ultrasound (EUS)-guided fine needle biopsies (FNB), assess take rates compared to surgery-derived PDXs, and demonstrate the histological and genetic resemblance to the original tumor.

Patients and methods: Biopsies of untreated pancreatic carcinoma were collected at surgery and during EUS and processed to generate PDXs.

Introduction

Treatment outcomes of pancreatic ductal adenocarcinoma (PDAC), the most common malignancy of the pancreas, have improved little over recent decades [1]. This is at least partly due to the lack of suitable preclinical in vivo models [2]. Recently, researchers have been able to establish xenografts in immunodeficient mice by implanting patient-derived tumor tissue obtained during surgery from various tumor types including PDAC. The main advantage of these xenografts is preservation of the original histological and genetic characteristics, even after consecutive passages. Additionally, these models have been shown to be predictive of clinical outcome and can be used for biomarker identification and personalized medicine [3]. However, large tissue quantities (10–30 mm³) are often needed to successfully generate patient-derived xenografts (PDXs), thereby limiting this technique to patients who are eligible for surgery [4].

As PDAC is usually diagnosed in a locally advanced setting, a substantial proportion of patients are excluded from curative surgery. Obtaining tumor material from these patients by an invasive procedure to generate xenograft models is irresponsible from a clinical perspective. Minimally invasive approaches that allow the establishment of xenografts at all clinical stages are urgently needed. In this pilot study we report the successful establishment of PDXs using fine needle biopsies (FNBs) obtained by endoscopic ultrasound (EUS) in patients with locally advanced disease.

Materials and methods

Collection of biopsy specimens

All procedures were approved by the Medical Ethical Committee (ML8713) and the Clinical Trial Center (S54185) of the University Hospitals of Leuven, Belgium in accordance with the most recent Helsinki Declaration. All patients provided signed informed consent for participation.

EUS-guided FNB specimens

Ten treatment-naive patients with a suspected malignant tumor of the pancreas on imaging were referred for EUS to obtain a tissue specimen for confirmation of carcinoma (Table 1). After...
Table 1 Clinical and pathological characteristics of the 14 patients with pancreatic carcinoma.

<table>
<thead>
<tr>
<th>Patient identifier</th>
<th>Biopsy technique</th>
<th>Sex</th>
<th>Age, years</th>
<th>Location in pancreas</th>
<th>Tumor size, cm</th>
<th>Pathology</th>
<th>Differentiation grade</th>
<th>TNM classification</th>
<th>Prior therapy</th>
<th>Survival from diagnosis, months</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAC001</td>
<td>Surgery</td>
<td>Male</td>
<td>55</td>
<td>Caput</td>
<td>Ø 2</td>
<td>Primary PDAC</td>
<td>Moderate</td>
<td>pT1 N0 M0</td>
<td>No</td>
<td>24</td>
</tr>
<tr>
<td>PAC002</td>
<td>Surgery</td>
<td>Male</td>
<td>61</td>
<td>Cauda</td>
<td>2 × 2 × 2.5</td>
<td>Primary PDAC</td>
<td>Moderate – poor</td>
<td>pT3 N1 M0</td>
<td>No</td>
<td>Lost to follow-up</td>
</tr>
<tr>
<td>PAC003</td>
<td>Surgery</td>
<td>Male</td>
<td>78</td>
<td>Caput</td>
<td>Ø 2</td>
<td>Primary PDAC</td>
<td>Poor</td>
<td>pT3 N1 M0</td>
<td>No</td>
<td>20</td>
</tr>
<tr>
<td>PAC004</td>
<td>EUS-FNB</td>
<td>Male</td>
<td>68</td>
<td>Corpus – cauda</td>
<td>Ø 3.8</td>
<td>Primary PDAC</td>
<td>Not defined</td>
<td>cT4 N1 M1</td>
<td>No</td>
<td>15</td>
</tr>
<tr>
<td>PAC005</td>
<td>Surgery</td>
<td>Female</td>
<td>46</td>
<td>Cauda</td>
<td>4.5 × 3.6 × 1.8</td>
<td>Primary PDAC</td>
<td>Moderate</td>
<td>pT3 N1 M0</td>
<td>No</td>
<td>23</td>
</tr>
<tr>
<td>PAC006</td>
<td>EUS-FNB</td>
<td>Female</td>
<td>84</td>
<td>Caput</td>
<td>2.1 × 1.8</td>
<td>Primary PDAC</td>
<td>Not defined</td>
<td>cT3 N0 M0</td>
<td>No</td>
<td>7</td>
</tr>
<tr>
<td>PAC007</td>
<td>EUS-FNB</td>
<td>Male</td>
<td>67</td>
<td>Corpus – cauda</td>
<td>Ø 7.3</td>
<td>Primary PDAC</td>
<td>Not defined</td>
<td>cT4 N1 M1</td>
<td>No</td>
<td>3</td>
</tr>
<tr>
<td>PAC008</td>
<td>EUS-FNB</td>
<td>Female</td>
<td>53</td>
<td>Corpus – cauda</td>
<td>Ø 8</td>
<td>Primary PDAC</td>
<td>Poor</td>
<td>pT3 N1 M1</td>
<td>No</td>
<td>8</td>
</tr>
<tr>
<td>PAC009</td>
<td>EUS-FNB</td>
<td>Male</td>
<td>79</td>
<td>Cauda</td>
<td>Ø 4</td>
<td>Primary PDAC</td>
<td>One out of two biopsies showed malignancy</td>
<td>cT4 N0 M0</td>
<td>No</td>
<td>17</td>
</tr>
<tr>
<td>PAC010</td>
<td>EUS-FNB</td>
<td>Male</td>
<td>84</td>
<td>Corpus – cauda</td>
<td>4.3 × 3.2 × 3.1</td>
<td>Primary PDAC</td>
<td>Not defined</td>
<td>cT4 N1 M1</td>
<td>No</td>
<td>3</td>
</tr>
<tr>
<td>PAC011</td>
<td>EUS-FNB</td>
<td>Male</td>
<td>65</td>
<td>Cauda</td>
<td>6.5 × 4.8 × 4.5</td>
<td>Primary acinar cell carcinoma</td>
<td>Not defined</td>
<td>cT4 N1 M0</td>
<td>No</td>
<td>17</td>
</tr>
<tr>
<td>PAC012</td>
<td>EUS-FNB</td>
<td>Female</td>
<td>61</td>
<td>Corpus – cauda</td>
<td>4.2 × 1.9</td>
<td>Primary PDAC</td>
<td>Necrotic tissue with limited amount of cells</td>
<td>cT4 N0 M1</td>
<td>No</td>
<td>7</td>
</tr>
<tr>
<td>PAC013</td>
<td>EUS-FNB</td>
<td>Male</td>
<td>64</td>
<td>Caput</td>
<td>Not defined</td>
<td>Primary PDAC</td>
<td>Necrotic tissue with limited amount of cells</td>
<td>cT4 N1 M1</td>
<td>No</td>
<td>Lost to follow-up</td>
</tr>
<tr>
<td>PAC014</td>
<td>EUS-FNB</td>
<td>Male</td>
<td>58</td>
<td>Cauda</td>
<td>Not defined</td>
<td>Primary PDAC</td>
<td>Not defined</td>
<td>cT4 N1 M1</td>
<td>No</td>
<td>10</td>
</tr>
</tbody>
</table>

EUS-FNB, endoscopic ultrasound-guided fine needle biopsy; PDAC, pancreatic ductal adenocarcinoma.
the tumor had been located using a linear Pentax echoendoscope on an Avius Hitachi platform, FNBs were performed using a 22G ProCore needle (Cook Endoscopy, Winston-Salem, North Carolina, USA). Samples were processed for routine cytological and diagnostic evaluation or were inoculated into sterile Roswell Park Memorial Institute (RPMI) 1640 transport medium (Life Technologies) supplemented with antibiotics (penicillin-streptomycin 100 U/mL and 100 µg/mL, respectively; gentamicin 50 µg/mL; Fungizone 1 µg/mL [Life Technologies]).

**Surgical biopsy specimens**

Surgical biopsies from four patients with confirmed pancreatic adenocarcinoma and no evidence of locally advanced or metastatic disease were obtained at the time of tumor resection (Table 1). The surgical biopsy was placed in transport medium and prepared for engraftment.

**Establishment of patient-derived PDAC xenografts**

Female NMRI nude mice (aged 6–8 weeks; Taconic, Denmark) were housed in individual ventilated cages in specific pathogen-free (SPF) conditions. All research procedures were executed in accordance with the applicable legal guidelines and under approval of the medical ethical committee for laboratory animals of the KU Leuven (P147/2012).

To facilitate the FNB implantation, a tissue pellet was made by centrifugation (Eppendorf Centrifuge 5810R) for 10 minutes at 1000 rpm. The interscapular fat pad of the mouse was externalized and a small pocket was created using an aseptic surgical technique. A small surgically resected tumor piece of 8 mm³ was placed in the pocket or a tissue pellet obtained via EUS. Tumors were harvested as soon as their maximum volume reached 1000–1500 mm³ and were re-transplanted into the next generation (F1, F2, F3, F4, and F5). Engraftment time was defined as the time between pellet or biopsy implantation and tumor harvest at maximum volume. Tissue from each tumor was stored for histological analysis, molecular profiling, and cryopreservation.

**Histology and immunohistochemistry**

Hematoxylin and eosin (H&E) staining was performed on formalin-fixed paraffin-embedded (FFPE) tumor samples to assess the general tumor morphology including the pattern of growth, grade, and specific cyto-architectural characteristics. Immunohistochemistry (IHC) was performed on F1 and F3 tumors specifically to study the vascular composition of the stroma and to assess the overall preservation of the tumor phenotype.

**High-throughput sequencing**

Whole-exome sequencing (WES) was performed as described previously [5]. DNA libraries were prepared using the KAPA DNA Library Preparation Kit (KAPA Biosystems Inc.), after which exonic fragments were captured (SeqCap EZ Human Exome Library; Roche) and sequenced (HiSeq2000; Illumina) at a coverage >30×. Raw sequencing reads were mapped to both human (NCBI37/hg19) and mouse (GRCm38/mm10) reference genomes using Burrows–Wheeler Aligner (BWA) [6] and processed with the Genome Analysis Toolkit [7]. Exonic non-synonymous mutations with a coverage <5× (germline DNA) and >10× (primary or xenograft tumor) were selected. Cancer consensus genes described by Catalogue of Somatic Mutations in Cancer (COSMIC) were used to compare between primary and corresponding xenograft tumors [8].

Whole-genome low-coverage (shallow) sequencing

DNA was prepared using the KAPA DNA Library Preparation Kit (KAPA Biosystems Inc.) and sequenced at low coverage on a HiSeq2000. Raw sequencing reads were mapped to the human reference genome (NCBI37/hg19) using BWA, revealing on average 5 897 670 mapped reads. Copy-number alterations (CNAs) were identified by QDNAseq v.1.0.5 by binning the reads in 100-kb windows [9] and segmenting the bin values by ASCAT v.2.0.7 [10].

**Targeted re-sequencing of KRAS and TP53**

Xenografts with no matched germline DNA were analyzed for KRAS mutations using Sequenom MassArray [11]. TP53 mutations were detected using targeted re-sequencing. Amplicons for TP53 exons were amplified using PCR, subjected to KAPA DNA Library Preparation, and sequenced on a HiSeq2000 with a cover-

---

**Table 2** Patient-derived tumor xenograft characteristics.

<table>
<thead>
<tr>
<th>Patient identifier</th>
<th>Success rate in F1 *</th>
<th>Engraftment time F1, weeks</th>
<th>Engraftment time F3, weeks</th>
<th>Differentiation grade F3</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAC001</td>
<td>2/4</td>
<td>25</td>
<td>9</td>
<td>Moderate</td>
</tr>
<tr>
<td>PAC002</td>
<td>1/4</td>
<td>16</td>
<td>12</td>
<td>Moderate to poor</td>
</tr>
<tr>
<td>PAC003</td>
<td>3/4</td>
<td>11</td>
<td>8</td>
<td>Poor</td>
</tr>
<tr>
<td>PAC004</td>
<td>1/1</td>
<td>20</td>
<td>7</td>
<td>Moderate</td>
</tr>
<tr>
<td>PAC005</td>
<td>3/4</td>
<td>24</td>
<td>16</td>
<td>Moderate</td>
</tr>
<tr>
<td>PAC006</td>
<td>1/2</td>
<td>21</td>
<td>5</td>
<td>Well to moderate</td>
</tr>
<tr>
<td>PAC007</td>
<td>1/1</td>
<td>13</td>
<td>8</td>
<td>Moderate to poor</td>
</tr>
<tr>
<td>PAC008</td>
<td>0/1</td>
<td>Not engrafted</td>
<td>Not engrafted</td>
<td>NA</td>
</tr>
<tr>
<td>PAC009</td>
<td>0/1</td>
<td>Not engrafted</td>
<td>Not engrafted</td>
<td>NA</td>
</tr>
<tr>
<td>PAC010</td>
<td>2/2</td>
<td>8</td>
<td>5</td>
<td>Poor</td>
</tr>
<tr>
<td>PAC011</td>
<td>1/1</td>
<td>27</td>
<td>8</td>
<td>Well</td>
</tr>
<tr>
<td>PAC012</td>
<td>0/1</td>
<td>Not engrafted</td>
<td>Not engrafted</td>
<td>NA</td>
</tr>
<tr>
<td>PAC013</td>
<td>0/2</td>
<td>Not engrafted</td>
<td>Not engrafted</td>
<td>NA</td>
</tr>
<tr>
<td>PAC014</td>
<td>2/2</td>
<td>14</td>
<td>5</td>
<td>Poor</td>
</tr>
</tbody>
</table>

*Number of mice with engrafted tumor/total number of mice.

NA, Not applicable.

---

Hermans Els et al. EUS-guided FNB-derived pancreatic xenografts ... Endoscopy
age >500x. Illumina and target-specific primers were removed using FastqMCF [12]. Further analysis was performed in a manner similar to the WES analysis.

Statistics
All statistics were performed using SPSS Statistics v22 (IBM). Between-group differences in engraftment times were assessed using the Mann–Whitney U test. Spearman correlation coefficients were calculated to investigate the link between engraftment times and patient survival.

Results
Engraftment rates of PDXs derived from FNB and surgery
In this study, we included 14 pancreatic tumors. All tumor and PDX characteristics are summarized in Table 1 and Table 2. Overall, the engraftment rate was 71% (10/14). All surgical samples were successfully engrafted (4/4), whereas an engraftment rate of 60% (6/10) was observed for the FNBs. The average engraftment time for all biopsies was 17.9 weeks at F1 and 8.3 weeks at F3 (Table 2). FNB-derived PDXs had a tendency towards faster engraftment at F1 (17.2 vs. 19 weeks; \( P=0.67 \)), a difference that became statistically significant at F3 (6.3 vs. 11.3 weeks; \( P=0.02 \)). Of note, there were progressive correlations between patient survival time and general engraftment time at F1 (\( r=0.58; P=0.1 \)) and at F3 (\( r=0.71; P=0.03 \)).

Preservation of tumor morphology and changes in the stromal component
At the histological level, the morphology, epithelial origin, and differentiation grade of all of the PDAC xenografts was maintained over different transplant generations. No differences could be observed between xenografts derived from EUS (Fig. 1a) and from surgery (Fig. 1b), on either H&E staining or CK7 immunostaining (Fig. 1c).

![Fig. 1](image-url) Engrafted pancreatic ductal adenocarcinoma (PDAC) patient-derived tumor xenografts (PDX) models maintain the original tumor morphology and epithelial origin irrespective of the biopsy technique (stains: a,b, hematoxylin and eosin [H&E]; c, CK7; magnification × 20; scale bar 50 µm). a Histology of a fine needle biopsy (FNB) from a PDAC in the corpus of the pancreas (top image; patient identifier PAC004) showing a background of granulocytes and red blood cells with multiple clusters of atypical tubular glandular structures lined by columnar cells often characterized by hyperchromatic pleomorphic nuclei and mucin production. The first passage (middle image) and third passage (bottom image) of the FNB-derived PDX retains a tubular glandular architecture with mucus-secreting cells and focal nuclear atypia. b Histology of a surgically resected neoplasia of the cauda of the pancreas, which was postoperatively diagnosed as a moderately differentiated PDAC (top image; PAC005), showing irregular micropapillary and cribriform glandular structures composed of mucin-producing cells embedded in desmoplastic stroma. The first passage (middle image) and third passage (bottom image) of the PDX show similar glandular structures lined by mucus-secreting columnar cells with polarized nuclei present. c A poorly differentiated PDAC stained by the epithelial marker CK7 shows diffuse expression in the epithelial component of the original specimen (top image; PAC003), with similar expression shown on the third passage of the xenograft (middle image). A similar appearance is seen in the third passage (lower image) of an FNB-derived xenograft from a patient with a poorly differentiated PDAC (PAC007).
The striking desmoplastic stromal reaction, evident in all original tumor biopsies via Masson Trichrome staining, disappeared in the first passage of the PDXs (Fig. 2a). Human-derived fibroblasts were replaced with loosely arranged murine-derived fibroblasts as demonstrated by loss of human vimentin expression from F1 onwards (Fig. 2b). In addition, tumor neovascularization by murine vessels, confirmed by anti-mouse CD31 staining, was already present in F1 (Fig. 2c).

The invasive character of the tumor, as indicated by perineural and local invasion into the adipose tissue and fascial planes, also included CK7-positive emboli in peritumoral lymphatic vessels and human vimentin expression in the neoplastic cells. The latter expression suggests activation of epithelial-to-mesenchymal transition (EMT); however, no distant metastases were detected.

**Preservation of genetic alterations**

When analyzing WES data for the F3 xenografts (n=6), we observed on average 79±27 somatic non-synonymous or stop – gain mutations in each xenograft. Interestingly, we observed several mutations in genes known to be frequently mutated in PDAC, including \textit{KRAS}, \textit{TP53}, \textit{CDKN2A}, and \textit{SMAD4} [13]. Because \textit{TP53} and \textit{KRAS} are by far the most frequently mutated genes in PDAC, we assessed the mutation status of both of these genes in all of the other xenografts using targeted re-sequencing. By combining both WES and targeted sequencing data, we found that \textit{TP53} and \textit{KRAS} were mutated in 60% and 90% of tumors, respectively. Among these, xenografts from surgery and from FNB both contained frequent mutations in \textit{KRAS} (5/6 in FNB-derived vs. 4/4 in surgery-derived xenografts; \(P>0.05\)) and \textit{TP53} (3/6 for FNB-derived vs. 3/4 for surgery-derived xenografts; \(P>0.05\)), which is similar to what has been observed by others [13].
By profiling somatic copy number changes using low-coverage whole-genome sequencing and comparing between xenografts derived from FNB and from surgery, we found that the average ploidy was 2.7±0.9 and 2.8±0.8, respectively. We observed an almost identical number of breakpoints (57±12 for FNB-derived vs. 54±17 for surgery-derived xenografts) and percentage of the genome affected by copy number changes (59±14 % for FNB-derived vs. 57±20% for surgery-derived xenografts).

When comparing CNAs between primary tumors and surgery-derived xenografts, we found that 88% (patient identifier PAC001), 67% (PAC003), and 68% (PAC005) of the genome had an equal copy number. Also CNAs affecting KRAS and TPS3 were common, being found in seven and eight of the eight xenografts, respectively, with no difference between FNB-derived and surgery-derived PDXs (data not shown).

Finally, we found that the majority of somatic mutations (n=50) detected by WES in PAC005, in particular those affecting cancer consensus genes (n=4), were present in both primary and xenograft tumors (Fig. 3), and that only a limited number of mutations were present in only the primary tumor (n=13) or xenograft (n=19). Surgery-derived xenografts were therefore very similar in comparison to the original tumors when assessed by WES and copy number profiling.

Discussion

In this study we report the successful engraftment and establishment of PDX models using tissue obtained by EUS-guided FNB in patients with pancreatic carcinoma. To achieve this, we developed an engraftment protocol that included an additional centrifugation step allowing the implantation of a 22G EUS-acquired tissue micro-core. We showed successful engraftment in 60% (6/10) of cases. This engraftment rate is similar to that described previously for surgical specimens [2,14,15].

To further explore the clinical relevance of PDXs established using FNB samples, we compared the tumor morphology from the first passage to the third using histology and immunohistochemistry. The specific PDAC PDX-related features, such as architecture and stromal content, were also confirmed in xenografts derived from FNBs [16]. Specifically, tumor morphology and differentiation grade were maintained upon propagation, while a general loss of stroma was observed, as was also demonstrated in surgically acquired PDAC PDXs and other tumor types. Human stroma was substituted by loose murine fibroblasts and vascular murine tissue from the first generation onwards [15].

The small differences in mutations and CNAs between primary tumors and xenografts that we observed are probably attributable to intra-tumor heterogeneity within the primary tumor and continued evolutionary pressure, which results in the accumulation of novel mutations in the xenografts. Overall, no obvious genetic differences were observed between the primary tumor and the xenografts from surgically derived biopsies, or between the xenografts derived from FNB and from surgery. Despite the limited number of PDXs, our data strongly suggest that FNB-derived xenografts bear similar genetic characteristics to their original primary tumor and may therefore be used, like surgically derived xenografts, in personalized medicine and drug development.

Competing interests: Schalk van der Merwe is on the advisory boards of Cook Endoscopy and Boston Scientific and holds a Chair in Interventional Endoscopy supported by an educational grant from Cook Endoscopy.

Institutions

1 Laboratory of Gynecologic Oncology, Department of Oncology, University of Leuven, Leuven, Belgium
2 Department of Gastroenterology and Hepatology, University of Leuven, Leuven, Belgium
3 Laboratory of Hepatology, Department of Clinical and Experimental Medicine, University of Leuven, Leuven, Belgium
4 Laboratory for Translational Genetics, Department of Oncology, University of Leuven, Leuven, Belgium
5 VIB, Vesalius Research Center, Leuven, Belgium
6 Division of Translational Cell & Tissue Research, Department of Imaging and Pathology, University of Leuven, Leuven, Belgium
7 Department of Abdominal Surgery, University of Leuven, Leuven, Belgium
8 Antoni van Leeuwenhoek, Nederlands Kanker Instituut, Centrum Gynaecologische Oncologie Amsterdam (CGOA), Amsterdam, The Netherlands
Acknowledgments

The authors thank all the collaborators of Trace involved in the establishment of the PDX models: Debby Thomas, Ellen Gommé (KU Leuven); Lieve Verbiest (UZ Leuven). Trace (www.uzleuven-kuleuven.be/lki/trace) is a member of the European Consortium (EurOPDX; www.europdx.eu). Frédéric Amant is senior researcher for the Research Fund Flanders (FWO). Immunohistochemical stains were in part provided by InfraMouse (KU Leuven-VIB) through a Hercules type 3 project (ZW09–03). This work is supported by the Ministries of Health, Belgium Cancer Plan.

References
7 McKenna A, Hanna M, Banks E et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res 2010; 20: 1297–1303
9 Scheinin I, Sie D, Bengtsson H et al. DNA copy number analysis of fresh and formalin-fixed specimens by shallow whole-genome sequencing with identification and exclusion of problematic regions in the genome assembly. Genome Res 2014; 24: 2022–2032
12 Aronesty E. Comparison of sequencing utility programs. The Open Bioinformatics Journal 2013; 7: 1–8