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A feasibility study characterising tumour microenvironment of longitudinally collected biopsies of pancreatic tumours upon endoscopic ultrasound guided radiofrequency ablation

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Highlights

- First EUS-RFA clinical study to longitudinally collect fine-needle aspiration biopsy (FNAB) and profile PDAC microenvironmental transcriptome changes
- The immuno-modulatory role, including change in checkpoint genes, of RFA-treated samples depends on baseline FNAB molecular features
- Established FNAB-derived CAF culture and bulk tumour profiles demonstrated stromal and cancer subtype remodelling in post-treated samples
- Classical PDA subtype genes are downregulated, and other subtype genes have no change upon RFA treatment
- RFA post-chemotherapy offers a potential avenue for personalised combination anti-checkpoint immunotherapy strategy

Abstract

Background

Most patients with pancreatic ductal adenocarcinoma (PDAC) are metastatic at presentation with dismal prognosis warranting improved systemic therapy options. Longitudinal sampling for assessment of treatment response poses a challenge for validating novel therapies. In this feasibility study, we evaluate the role of endoscopic ultrasound (EUS)-guided serial fine-needle aspiration biopsies (FNABs) to study the molecular changes associated with radiofrequency ablation (RFA).

Methods

Two stage III inoperable gemcitabine-treated PDAC patients were recruited in the treatment arm of ARDEO (ethically approved Phase-II prospective randomized clinical study). Post examination, targeted RFA was delivered thrice, and sequential FNABs of tumour were taken before and after treatment and analysed using a custom NanoString panel (144 genes) consisting of cancer and cancer-associated fibroblast (CAFs) subtypes and immune changes. CAF culture was established from one FNAB and characterised by immunofluorescence and immunoblotting.

Results

Serial EUS-RFA treatments were well tolerated with no complications. Both patients had stable disease immediately after EUS-RFA, however, with different survival outcomes. Two-course RFA led to upregulation of *CD1E* gene (involved in antigen presentation) in both, patient 1 and 2 (4.5 and 3.9-fold) compared to baseline. Patient 1 showed increased T cell genes (*CD4* – 8.7-fold, *CD8* – 35.7-fold), cytolytic function (6.4-fold) and inflammatory response (8-fold). Greater than 2-fold upregulation of immune checkpoint genes was observed post 2nd RFA in patient 1, patient 2 or both. Further, two-course RFA led to increased *PDGFRα* (4.5-fold) and CAF subtypes B and C genes in patient 1 and subtypes A, B and D genes in patient 2. Patient2-derived CAFs post 1st RFA showed expression of *PDGFRα*, *POSTN* and *MYH11* proteins. Finally, RFA led to downregulation of classical PDA subtype in both patients.

Conclusions

For the first time, this feasibility study validates longitudinal sampling by EUS-FNABs as an appropriate research tool to study tumour microenvironmental changes associated with local PDAC immunomodulatory treatments like RFA.

Keywords

EUS-RFA, FNAB, PDAC, tumour microenvironment, immunotherapy, tumour microenvironment, cancer subtypes, CAF subtypes, cancer-associated fibroblast, immune checkpoint genes.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the third most common cause of cancer death after colorectal and lung cancer (Ansari et al. 2016), with a five-year survival rate of <5% despite significant advances in cancer management for other solid organ malignancies over the last few decades. At presentation, only 5-25% of patients are eligible for radical curative surgery and, even in these patients, only 30% of patients will survive five years. At diagnosis, ~40% of patients have locally advanced, unresectable, stage 3 disease, usually due to significant encasement of the superior mesenteric vein/portal vein or involvement of the superior mesenteric or hepatic arteries. The prognosis for this group of patients is extremely poor, with a median survival of 12-14 months on chemotherapy. While a small proportion of these patients respond well to chemotherapy and can subsequently undergo surgery, palliative chemotherapy remains the only treatment option in most patients (Gillen et al. 2010).

Radiofrequency ablation (RFA) describes destruction of the tumour using heat generated by a high frequency alternating current applied through an electrode tip. The tissue is heated to 60°C, resulting in coagulative tissue necrosis at the center of the ablation zone. RFA is used to treat a few solid organ malignancies and is routinely used in both primary and secondary hepatocellular carcinoma (HCC) (Livraghi et al. 2000; M Pai et al. 2012; Madhava Pai et al. 2015; Weber et al. 2002). However, complete eradication of stage 3 PDAC is not usually possible with RFA due to tumour proximity to major vascular structures and the duodenum and a consequent risk of injury. Nevertheless, RFA of inoperable stage 3 PDAC through laparotomy has been found to be safe. Indeed, a recent large series examining the procedure

reported a median overall survival (OS) and disease-specific survival (DSS) of 20 and 23 months, respectively, with an RFA-related morbidity of 15% and overall mortality of 3%(Girelli et al. 2013), which compares to a median survival of 12-14 months on standard chemotherapy(Gillen et al. 2010).

Following RFA, the localised necrosis of the tumour initiates a cascade of events including release of proinflammatory signals, release of cellular debris representing a source of tumour antigens, and a host adaptive immune response against the tumour(Evrard et al. 2007). RFA appears to induce both innate and adaptive immune responses against the tumour through effective infiltration of dendritic cells, boosted antigen presentation, and an intensified T cell response(den Brok et al. 2006; Dromi et al. 2009; Haen et al. 2011; Napoletano et al. 2008; Zerbini et al. 2006). Recent studies have suggested that cancer-associated fibroblasts (CAFs), which are central components of the desmoplastic stroma found in most PDACs, can positively or negatively impact anti-tumour immune responses(Biffi et al. 2019; Kieffer et al. 2020; Neuzillet et al. 2019; Sahai et al. 2020). Others and we have recently reported heterogeneity in CAFs and identified different subtypes (subtypes A-B or inflammatory (i)CAF/myofibroblast (my)CAF/antigen presenting (ap)CAF) with distinct associations with immune cells(Elyada et al. 2019; Neuzillet et al. 2019). Moreover, cancer subtypes (classical, quasi-mesenchymal (QM) and exocrine-like) and similar subtypes of PDAC have been shown to be associated with immune/stromal changes and patient prognosis(Bailey et al. 2016; Collisson et al. 2011; Moffitt et al. 2015). Thus, a comprehensive understanding of the cell types within the tumour microenvironment (TME) is needed when assessing anti-tumour responses associated with RFA in which the immune system plays a critical modulatory role.

There have been no previous reports of longitudinal sampling of pancreatic tumours when treated with radiofrequency ablation in PDAC patients, which is necessary to understand the mechanism of action of the treatment. It is also unknown whether minimally invasive tissue sampling through endoscopic ultrasound (EUS)-guided fine needle aspiration biopsy (FNAB) is adequate for sampling tissues subjected to RFA for cellular and molecular analyses. This proof-of-concept study therefore aimed to determine the feasibility of studying the potential mechanism of action of RFA in pancreatic cancer through serial EUS-FNAB.

116 **Methods**

117 *Study Design*

118 Patients were recruited through the treatment arm of the pilot phase of the ARDEO trial (A
119 phase II prospective Randomised clinical study of endoscopic ultrasound guided
120 radiofrequency ablation for inoperable pancreatic ductal adenocarcinoma). This is a
121 prospective randomised clinical study (REC reference: 18/SW/0103) in patients with stage 3
122 inoperable adenocarcinoma of the pancreas starting gemcitabine chemotherapy with a 1:1
123 randomisation. Patients in the treatment group receive up to 3 endoscopic ultrasound
124 radiofrequency ablation procedures, each a month apart and sequential EUS FNABs were taken
125 during each procedure prior to RFA treatment. Written informed consent was taken from the
126 patients before enrolling into the study. Patients in the control group will receive best medical
127 care. A study flow diagram is illustrated in Fig 1a. PDAC patients in the treatment group were
128 recruited over 2 months at the Hammersmith Hospital, Du Cane Road, London.

129 *Endoscopic-ultrasound guided radiofrequency ablation*

130 Following EUS examination of the pancreas and primary tumour site, an FNAB of the tumour
131 was taken and then targeted RF delivered using 10 Watts for 2 minutes per application using
132 the Habib EUS RFA (Boston Scientific, MA, USA). For the second and third EUS
133 examinations, the ablation zone is examined, and a fine needle biopsy taken prior to RFA.
134 FNABs were snap frozen in liquid nitrogen and transported on dry ice. Routine biochemistry
135 tests including bilirubin and CA19-9 were analysed at Imperial College Healthcare NHS Trust
136 laboratory.

137 *Nucleic acid extractions from FNABs*

138 Flash frozen FNABs were homogenised in Precellys beaded tubes (Bertin Technologies™,
139 Montigny-le-Bretonneux, France) containing 700 µl of lysis buffer using a tissue homogeniser
140 (Bertin Technologies™, Montigny-le-Bretonneux, France). RNA from tumour lysates was
141 extracted using AllPrep DNA/RNA/miRNA Universal Kit (Qiagen™, Manchester, UK)
142 according to manufacturer's instructions. RNA yield was estimated by Nanodrop 2000
143 (Thermo Fisher Scientific™, Delaware, USA).

144

145

Transcriptomic profiling on NanoStringTM nCounter

100 ng of total mRNA was used to run a custom designed human gene panel encompassing cancer, immune and cancer associated fibroblast genes along with house-keeping reference genes for data normalization (144 genes, Table S1). Tumour samples were run on the nCounter® Max analysis system (NanoString Technologies, CA, USA) as per the protocol previously published by us (Ragulan et al. 2019). Data was assessed for quality, normalised to housekeeping genes and log₂ transformed using nSolver 4.0 software. Gene expression values were plotted using GraphPad Prism Version 8.0 (for Windows, GraphPad Software; La Jolla, CA, USA, www.graphpad.com).

Gene Scores and subtypes

Gene scores for cancer subtypes, T cell, B cell, cytolytic function and inflammation were obtained by averaging the expression of genes specific to the respective subtype, cell types or immune function (Forero et al. 2019; Fulkerson et al. 2004; Newman et al. 2015; Szabo et al. 2019; Weng, Araki, and Subedi 2012; Zheng et al. 2017). List of subtype and cell type specific genes used here to arrive at scores can be found in Table S2. CAF subtype scores were derived by averaging the expression of top differentially expressed genes representing pCAF subtypes (Neuzillet et al. 2019) for each sample. PDA subtypes (Collisson et al. 2011) were predicted by the nearest template prediction method (NTP) (Hoshida 2010) using subtype specific genes identified previously (Collisson et al. 2011).

Isolation of patient-derived cancer-associated fibroblasts from EUS-FNABs

Cancer associated fibroblasts from fresh FNABs were isolated by outgrowth method (Apte et al. 1998; Bachem et al. 1998). Briefly, tissue biopsies were transported in advanced Dulbecco's Modified Eagle Medium/Ham's F12 (Gibco, NY, USA) containing 10% fetal bovine serum (FBS, Qualified HI; Gibco, NY, USA) and 1% penicillin-streptomycin solution (Gibco, NY, USA) (complete media). FNABs were washed twice in phosphate buffered saline (PBS) containing 1% penicillin-streptomycin solution and cut into smaller pieces using sterile scalpels. Tissue digestion was performed in PBS containing 1mM ethylenediamine-tetraacetic acid (PBS-EDTA) along with 2x TrypLE (Gibco, NY, USA) and 20 µl of DNase enzyme for 1 h at 37°C. Following digestion, tubes were centrifuged for 5 min at 1200 rpm and the tissue pellets were resuspended in 2 ml of ACK lysis buffer (Gibco, NY, USA) to deplete red blood cells by incubating on ice for 3 min. 5 ml of complete media was added to neutralize the

solution and tubes were centrifuged for 5 min at 1200 rpm. 3 ml of pre-warmed complete media was added to the tissue pellet and contents were placed on 35 mm dishes. After a week, fibroblasts that grew out from the tissue bits were trypsinised and passaged once a week. Cells were maintained at 37°C in a 5% CO₂ incubator. Cells were screened for mycoplasma by polymerase chain reaction and found to be negative (Figure S1). DNA for STR profiling was sent to Eurofins Genomics (Wolverhampton, UK).

Immunofluorescence

2 x 10⁴ patient-derived CAFs were seeded on 8-well chambered coverslips (ibidi, Thistle Scientific, Glasgow, UK) in complete growth media. Next day, media was aspirated and cells were washed twice with cold PBS and fixed in 4% paraformaldehyde for 15 min at room temperature (RT). After two gentle washes in PBS, cell permeabilisation and blocking was performed using PBS containing 5% bovine serum albumin (BSA) + 0.3% Triton-X-100 for 1 h at RT. Cells were incubated with primary antibodies overnight at 4°C. On the following day, cells were washed thrice with PBS and incubated with secondary antibody (in PBS containing 1% BSA + 0.05% Tween-20) for 1 h at RT in a moist dark chamber. Cells were washed thrice with PBS and stained with 4',6-diamidino-2-phenylindole (DAPI) (1:1000 in PBS) for 10 min at RT. Coverslips were mounted on 50 µl of VectaShield mounting media (Vector Labs, Detroit, USA) and imaged using Zeiss LSM700 (Jena, Germany). Antibody information is listed in Table S3.

Immunoblotting

0.6 x 10⁶ patient derived CAFs were seeded in complete growth media in 60 mm dishes. Next day, media was aspirated, and cells were washed twice with cold PBS. 30 µl of cold NP40 lysis buffer (50mM Tris-HCL, 150mM sodium chloride, 1% NP40) containing phosphatase and protease inhibitors (Pierce, Life Technologies Corporation, CA, USA) was added to obtain protein lysates. Protein concentrations were determined using BCA method (Pierce, Life Technologies Corporation, CA, USA) and 10 µg of protein was fractionated onto 12% sodium dodecyl sulphate-polyacrylamide gels. Blotting was performed on polyvinylidene difluoride (PVDF) membranes for 1 h at 100 V. Membranes were blocked in PBS containing 5% BSA + 0.12% Tween-20 for 1 h followed by probing with primary antibodies, overnight at 4°C. Next day, membranes were washed and incubated with anti-mouse/rabbit secondary antibodies conjugated to horseradish peroxidase. Immunoreactive bands were visualised on Licor

208 Odyssey Fc (Cambridge, UK) using enhanced chemiluminescence (ECL, Merck Millipore,
209 Darmstadt, Germany). β actin was used a loading control. Antibody information is listed in
210 Table S3.

211 *Statistics*

212 To assess RFA-mediated transcriptome modulation in serial FNABs, log₂ transformed gene
213 expression values at T2 (after 1st RFA) and T3 (after 2nd RFA) were subtracted from T1
214 (baseline) and denoted as *difference*. 2[^] (*difference*) was then performed to calculate fold
215 change in gene expression over the course of RFA treatment.

216

217

218 **Results**

219 The first two patients recruited in the treatment arm of the ARDEO trial were included in this
220 feasibility study. All EUS-RFA procedures were uneventful with no observed clinical
221 complications.

222 *Patient 1*

223 Patient 1 was a 71-year-old female with no significant co-morbidities who presented with
224 obstructive jaundice and weight loss in November 2018. On presentation she had a bilirubin of
225 223 and cancer antigen (CA)19-9 of 503 and underwent endoscopic retrograde
226 cholangiopancreatography (ERCP) with metal stent insertion and EUS and biopsy of a mass in
227 the neck of the pancreas in early December 2018. The tumour was found to be an
228 adenocarcinoma involving the splanchnic vasculature and deemed inoperable with a
229 radiological staging of tumour (T) and nodal status (N) of T4N1. She was enrolled in the
230 ARDEO trial on 24th January 2019 and started gemcitabine chemotherapy that day. She
231 underwent EUS with RFA ablations on 13th Feb, 13th March and 17th April. There were no
232 complications or morbidity relating to the EUS or RFA treatment. However, she did suffer with
233 Grade 1 nausea related to gemcitabine treatment that was fully responsive to the anti-nausea
234 drug, domperidone. The patient elected to take a treatment break after cycle 6 of gemcitabine
235 as she was thinking of relocating and wanted to go away on holiday. At the time of stopping
236 treatment, she had stable disease radiologically and her CA19-9 had decreased from 4887 when

enrolled in the trial to 392 on the 25th of April, which was after her final EUS treatment (cycle 3). After cycle 3, the CA19-9 increased to 1324 at the end of cycle 6 and at the start of the treatment break. She re-presented in October with liver metastases, portal vein thrombosis and a CA19-9 of 503,293 and underwent one cycle of CAPOX (oxaliplatin and capecitabine) chemotherapy but sadly passed away on the 15th of November.

Patient 2

Patient 2 is a 62-year-old male with a background of Type 2 Diabetes Mellitus, hypertension and hypercholesterolaemia who presented in December 2018 with abdominal pain, weight loss and jaundice. He never had elevated tumour markers (CA-19-9) since diagnosis. On cross-sectional imaging, he was found to have mass in the neck/body of the pancreas which was invading the splanchnic vessels with EUS biopsy confirming a diagnosis of adenocarcinoma with a radiological staging of T4N2M0 (M – represents presence of metastasis). He was enrolled in the ARDEO study on 2nd of February 2019 and started first cycle of Gemcitabine on 4 days later. He underwent EUS, biopsy and then RFA on 6th of March, 3rd April and 10th of May. He subsequently completed 10 cycles of gemcitabine maintaining stable disease on cross-sectional imaging and then started chemo-radiotherapy (capecitabine and 20 Fractions) in February which was stopped in March 2020 due to the risk of coronavirus at the height of the pandemic in London. Fig. 1b shows representative ultrasound images of tumour at baseline, tumour and ablation site after 3 courses of EUS-RFA and 16 months post treatment of patient 2S. Updated imaging on 28th of August 2020 shows stable disease (Fig. 1b).

Feasibility of evaluating whether EUS-RFA treatment elicits an anti-tumour immune response in PDAC

To obtain insight into RFA mediated modulation of the TME, we performed transcriptomic profiling of FNABs collected longitudinally over three courses of RFA for the two patients enrolled in our proof-of-principle study. A schematic representation of sample collection and approaches is outlined in Fig. 2a. A low-cost custom NanoString panel was designed to include genes for PDA and CAF subtypes identified previously (Collisson et al. 2011; Neuzillet et al. 2019), along with markers to assess changes in the TME as a result of RFA treatment (Table S2). Initial unsupervised hierarchical clustering of the six tumour samples grouped T3 FNABs (after 2nd RFA) of both the patients together and showed enrichment of T cell markers, immune checkpoint genes and increased fibrosis observed as upregulated CAF markers. T3 samples were most similar to T2 (after 1st RFA) profile but only in patient 2. T1 (baseline) and T2 (after

1st RFA) from patient 1 clustered together and shared similar expression profiles to T1 (baseline) from patient 2, except the latter was highly enriched for T and B cell genes along with cytolytic function markers, Fig. 2b. This suggests that the baseline sample from patient 2 was already immune-enriched, which is not the case with patient 1.

We first assessed RFA-induced immunological changes resulting in an increased adaptive immune infiltration into the tumours. A high T (7.3-fold) and B (2.2-fold) cell score at T3 in patient 1 compared to baseline and T2 was observed. There was no change in T and B cell score in patient 2 at T2 and T3 compared to baseline (Fig. 2c; shows log₂ expression). Overall, the T and B cell changes vary in individual patients after RFA treatment. Ratio of cytotoxic CD8⁺ T cells by FOXP3⁺ regulatory T cells has been shown to have prognostic and predictive value in multiple cancer types (Asano et al. 2016; Goto et al. 2018; Suzuki et al. 2010). We assessed this ratio using *CD8A* and *FOXP3* gene expression and found greater than 3-fold upregulation in patient 1 at both T2 and T3 compared to baseline. This ratio, however, was lower in patient 2 after RFA treatment (Fig. 2d).

Further, genes associated with proteins that participate in antigen presentation to T cells via major histocompatibility complexes I and II (*CD1E* for MHC-I (Angenieux et al. 2000; Blumberg et al. 1995) and *HLADQA1* for MHC-II (Majumder, Gomez, and Boss 2006) were also highly upregulated upon RFA. There was a 4.5-fold and 5.3-fold increase in expression of *CD1E* and *HLADQA1* respectively at T3 time point in patient 1. *CD1E* expression in patient 2 at T3 was also found to be 3.9-fold greater than at baseline but a decreasing trend in *HLADQA1* was observed (Fig. 2e). However, we warrant further validation of MHC-I and -II using additional gene sets representing the complexes in the future.

Next, we investigated the different immune cell types that may have been recruited to the tumours because of RFA. Patient 1 showed RFA course-dependent upregulation in T cell markers – *CD4* (8.7-fold at T3) and *CD8* (35.7-fold at T3), along with a 3.7-fold increase in markers representing NK cells. Genes representing macrophages and mast cells showed less than 2-fold change in patient 1 with respect to RFA treatment. *CD4* expression and *CD8* expression showed 2-fold and 3.2-fold decrease at T3 compared to baseline in patient 2. The baseline levels of CD4 and CD8 were, however, higher in patient 2 compared to patient 1, suggesting that patient 2 was already inflamed with CD4 and CD8 T cells before RFA.

A reduction in transcript levels of genes representing macrophages (2.6-fold) and mast cells (3.7-fold) and no change in NK cell genes was observed in patient 2 post-RFA (at T3) compared to baseline (Fig. 2f). Further, apart from upregulated T cell markers, a 6.4-fold and 8-fold increase in cytotoxic T cell function score and inflammation score, respectively was observed at T3 compared to baseline in patient 1. Decrease in cytolytic function score (14.17-fold at T3) and a 2-fold increase in inflammation score was observed after 2 courses of RFA in patient 2 (Fig. 2g).

T cell infiltration after RFA treatment has been shown to be accompanied by an exhausted T cell phenotype marked by increased expression of immune checkpoint genes (Fei et al. 2020). We studied the expression of known immune checkpoint genes with agonists/antagonists currently in clinical trials or approved for clinical use in other cancers (Hodi et al. 2018; Larkin et al. 2019; Overman et al. 2017). After 2 courses of RFA (T3), patient 1 showed >2-fold increase in *CD274 (PDL1)*, *IDO1*, *TNFRSF18 (GITR)*, Fig. 2h. Also, a >2-fold upregulation in *PDCD1 (PD1)*, *IDO1*, *TNFSF18 (GITRL)* and *TNFRSF18 (GITR)* was observed in patient 2 at T3, shown in Fig. 2i. These suggests that both these patients may be amenable to anti-checkpoint treatment after 2 courses of RFA treatment.

Feasibility of evaluating whether RFA remodels the tumour stroma in PDAC

Recent studies have reported loco-regional coagulation and necrosis as a result of RFA resulting in remodelling of the TME (Evrard et al. 2007; Livraghi et al. 2000). We investigated if the molecular changes brought about by RFA reflected a switch in our previously identified pCAF assigner (pCAF; cancer-associated fibroblast) subtypes (Neuzillet et al. 2019). Data from both patients at T3 indicated a marked upregulation (4.5-fold; patient 1 and 8.5-fold; patient 2 respectively) of pan-CAF marker - *PDGFR α* . Patient 1 tumour also showed a 3.8-fold increase in *ACTA2 (α SMA)* after 2 courses of RFA, Fig. 3a. RFA led to a modest increase in subtype B and C CAFs in patient 1, Fig. 3b. This is supported by our previous finding suggesting enhanced immune cell responses in pCAF subtype C-dominated tumours (Neuzillet et al. 2019), similar to inflammatory CAF subtype identified by Ohlund *et al.* (Öhlund et al. 2017). In contrast, RFA treated patient 2 tumour (T3) showed a 3.6-fold, 9-fold and 5-fold upregulation of pCAF subtypes A, B and D, respectively (Fig. 3c). We then selected the genes that have been previously validated by immunohistochemistry to exclusively represent pCAF subtypes and found an enrichment of *POSTN* (subtype A), *MYH11* (subtype B) and *PDPN* (subtype C) in both patients after RFA, however, the increase was pronounced particularly in patient 2

tumour at T2 and T3, Fig. 3d. In order to validate CAF-specific expression of these markers, we established cultures from patient FNABs and successfully obtained CAFs from patient 2 tumour at T2. A strong expression of pan-CAF markers - PDGFR α , α SMA and VIM was observed along with expression of subtype A (POSTN) and subtype B (MYH11) in the cultured CAFs (Fig. 3e).

Feasibility of evaluating whether RFA remodels the cancer subtypes of PDAC

In order to study how the crosstalk between immune cells and CAFs in the TME regulates cancer cells, we performed PDA subtyping of tumours pre- and post-RFA by NTP using subtype specific genes described previously (Collisson et al. 2011). Our data indicated that both patients at baseline were categorised into the classical PDA subtype. RFA led to a marked decrease in genes representing classical PDA subtype in both patients at T3 compared to baseline and T2, Fig. 3f and 3g. No particular change in exocrine-like or quasi-mesenchymal PDA subtype was observed in both the patients upon RFA. Overall, the concomitant enrichment of adaptive immune response and immune promoting CAF subtype suggest RFA mediated remodelling of stroma in PDAC.

Discussion

This feasibility study established the value of longitudinal sampling with EUS-FNABs to study local TME changes occurring in response to RFA in two patients. We successfully isolated RNA from these biopsies to comprehensively profile immune, CAF, and cancer cell gene expression using a panel of genes associated with PDAC subtypes (both cancer and CAF) and immune cell types. Using inferential statistics, we established different patterns of longitudinal remodeling of immune cells and stroma after RFA in these two patients, enrolled in the ARDEO trial, were treated with RFA, and who differed in terms of survival (not necessarily associated with RFA), treatment response, and change in CA19-9 and bilirubin levels. While both patients had stable disease after RFA treatment, one patient died due to other reasons. This study clearly elucidates heterogeneity in response to RFA in different patients depending on their baseline immune repertoire. This study paves the way to access samples longitudinally during treatment in clinical trials with EUS-RFA (to our knowledge for the first time) to assess the molecular changes and subtypes associated with responses to therapy.

We detected changes in genes participating in antigen presentation and T and B cell scores in response to RFA in one patient. Similar results have been reported in a rat model of HCC, which showed that necrotic cells release factors for several hours to days after RFA treatment leading to activation and maturation of dendritic cells. This further stimulates CD4⁺ and CD8⁺ effector T cells directed against the antigen MHC class I–dominant, class I–subdominant, and class II–dominant epitopes (Schueller et al. 2004). Indeed, in mice, similar MHC changes and T cell responses were found in urothelial carcinomas partially treated with RFA. Furthermore, animals with eradication of the primary tumour resisted further challenge with tumour implants, implying a durable immunological memory response(Dromi et al. 2009).

Our observation of increased cytolytic function and inflammatory gene expression after RFA in one patient corroborate with multiple studies in animals. For example, den Brok and colleagues elegantly demonstrated in mice that a significant proportion of draining lymph nodes contained tumour antigen following RFA than after vaccination. Furthermore, they showed that a combination of *in situ* tumour ablation and checkpoint inhibitor (anti-CTLA-4; depletion of regulatory T cells) therapy enhanced CD8⁺ T cell activation(den Brok et al. 2006). Interestingly, in the neoadjuvant setting in a murine model, RFA treatment demonstrated systemic immunity against cancer cells leading to better survival. This study suggests a potential application of RFA in patients with tumour types associated with increased risk of recurrence(Ito et al. 2015).

In a clinical trial of non-small cell lung cancer (NSCLC), Schneider and colleagues demonstrated local and systemic increase in CD4⁺ and CD8⁺ T cells after neoadjuvant RFA (followed by surgery) in patients. These increased T cells in resected tissues were noticed around the boundary where RFA was applied. They concluded that treatment of NSCLC with neoadjuvant RFA develops necrosis and provides long-term immunity against the tumour by activating T and dendritic cells(Schneider et al. 2016).

Our observation of enrichment of immune checkpoint genes in post-RFA treated tumours is consistent with a study by Shi et al., who found that RFA treatment of metastatic colorectal cancer showed increased PD-L1 expression and T cells in tumour tissues in a retrospective case controlled study(Shi et al. 2016). This provides a mechanism by which tumours can evade immune response and a potential target for combination therapy. Indeed, combined RFA and a CTLA-4 inhibitor (tremelimumab) was effective in a phase 1 trial of HCC patients. Those patients with clinical benefit from combination treatment of RFA and tremelimumab showed

enhanced CD8⁺ T cells(Duffy et al. 2017). Further, in a study of patients undergoing RFA for locally advanced PDAC, Giardino and colleagues showed longitudinally enhanced adaptive immunity associated with CD4⁺, CD8⁺, effector memory T cells and dendritic cells(Giardino et al. 2017), suggesting potential opportunity to target PDAC with a combination of RFA and immune checkpoint inhibitor(Shi et al. 2016).

Co-enrichment of cytotoxic immune infiltrates with pCAF subtypes B and C in post-RFA patient 1 FNAB confirms our previous finding, along with other studies, of immune cell enrichment in PDACs dominated by subtype B/C CAFs or iCAF(Ellyada et al. 2019; Helms, Onate, and Sherman 2020; Neuzillet et al. 2019). Successfully established patient-derived CAFs from limited FNAB material confirmed the expression of the pCAF subtype A and B marker, as found in the bulk tumour sample. Patient-derived cancer and CAF cultures from the extended ARDEO study cohort will further establish the consequences of RFA on this crosstalk with a view on developing combination treatment strategies.

Nevertheless, it is important to note that patient 2 had increased anti-tumour immune infiltrate at baseline itself, potentially responsible for prolonged overall survival. RFA courses led to no particular increase in T and B cells or cytolytic activity. Interestingly, immune checkpoint genes were upregulated upon RFA in patient 2. The overall immune milieu supports that patient 2 may have been a contender for immune checkpoint therapy. Further, response to RFA in the two patients was found to be heterogeneous providing an opportunity to obtain deeper insights into immune signaling in pre- and post-RFA samples. In addition, how these changes are associated with prior treatment, like gemcitabine, may be addressed in the larger sample set which is part of the ARDEO trial.

Both the patients at baseline showed enrichment of classical PDA subtype genes which were dramatically downregulated over the RFA courses. However, there was no drastic switch in the subtype to QM or exocrine-like, suggesting that RFA may be controlling cancer cells from switching to poor prognostic cancer type.

The results from this feasibility study need to be validated in the larger ARDEO cohort and other datasets of PDAC patients treated with RFA. Although these analyses suggest RFA-mediated change in immune, CAF, and cancer cell gene signatures, the biological implication of their presence and spatial distribution requires deeper investigation. Despite the limitations of these data from two patients and only 144 genes, this is the first feasibility study to report

transcriptomic profile of the TME during RFA treatment by longitudinal sampling using serial EUS-FNABs in PDAC patients.

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591 Figure Legends

592 Figure 1. EUS-RFA in human PDAC. a. Schematic representation of ARDEO study cohort,
593 b. Representative ultrasound images of the tumour and ablation site at baseline and after RFA
594 treatment. SOC – Standard of Care; CT – Computerised Tomography; RECIST - Response
595 Evaluation Criteria in Solid Tumours; FNAB – Fine Needle Aspiration Biopsy.

596 Figure 2. RFA induces immunogenicity in PDAC patients. a. Schematic illustration of
597 longitudinal FNABs and RFA courses along with approaches used in this study, b. Heatmap
598 depicting distribution of genes across samples and grouped by unsupervised hierarchical
599 clustering. Scale bar indicates log₂ normalised expression ranging between -1 and +1. Colour
600 bars represent appropriate CAF and PDA subtype-specific colours, c. Change in T and B cell
601 scores at T1, T2 and T3 in patient 1 and 2 respectively, d. Ratio of *CD8A/FOXP3* in patient 1
602 and 2. e. Change in expression of genes participating in antigen presentation via MHC-I and II
603 at T1, T2 and T3 in patient 1 and 2 respectively, f. Heatmap representing RFA-mediated
604 regulation of genes associated with different immune cell types; single scale bar for both
605 patients depicting log₂ normalised expression, g. Tumour-immune response in pre- and post-
606 RFA treated FNABs measured as cytolytic function and inflammatory score, h. and i.
607 Upregulation of immune checkpoint genes as a result of RFA in patient 1 and 2 respectively.
608 # represents greater than equal to two-fold change in gene expression between T1 and T3.
609 Colour key represents T1 in red, T2 in blue and T3 in cyan.

610 Figure 3. RFA remodels the TME. a. Dot plot showing upregulation of pan-CAF markers -
611 *PDGFRα* and *ACTA2* (*αSMA*) as a response to RFA in patient 1 and 2 respectively, b. and c.
612 Heatmaps representing a switch in the CAF subtypes over the course of RFA in patient 1 and
613 2 respectively; separate scale bars for the two patients depicting averaged log₂ normalised
614 subtype-specific CAF gene expression, d. Heatmap representing change in IHC validated
615 exclusive pCAF subtype-specific genes over the course of RFA in patient 1 and 2, e. Brightfield
616 (5x magnification) and confocal images of immunofluorescence staining of different CAF
617 markers in patient 2 T2-derived CAFs. Confocal images taken at 20x magnification; scale bar
618 represents 50 μm, f and g. Heatmaps representing a switch in the PDA subtypes over the course
619 of RFA in patient 1 and 2 respectively; colour bars represent NTP-derived PDA subtype and
620 separate scale bars for the two patients depicting averaged log₂ normalised gene expression
621 associated with each PDA subtype.

Figure 1

Figure 2

a. PDAC patients; n=2

b.

c.

d.

e.

f.

g.

h.

i.

Key

Figure 3

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