Intra-muscular transplantation improves engraftment rates for esophageal patient-derived tumor xenografts

Matthew Read, MBBS^{1,2}, David Liu, MBBS, BMedSci^{1,2}, Cuong P. Duong, MBBS, FRACS, PhD^{1,3}, Carleen Cullinane PhD^{1,2}, William K Murray, MBBS, FRCPA, FRCPATH⁵, Christina Fennell BBiomedSc(hons)^{1,2}, Jake Shortt, BMedSc, MBChB, FRACP, FRCPA, PhD^{2,6,7}, David Westerman, MBBS, FRACP, FRCPA, FFSc^{5,8}, Paul Burton, MBBS, FRACS, PhD^{9,10}, Nicholas J. Clemons, BSc(hons), PhD^{1,2,4*} and Wayne A. Phillips, BSc(hons), PhD^{1,2,3,4} * (*co-senior authors)

¹Surgical Oncology Research Laboratory, Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia; ²Sir Peter MacCallum Department of Oncology, University of Melbourne, Melbourne, Victoria, Australia; ³Division of Cancer Surgery, Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia; ⁴University of Melbourne Department of Surgery, St. Vincent's Hospital, Melbourne, Victoria; ⁵Department of Pathology, Peter MacCallum Cancer Centre, Melbourne, Australia; ⁶School of Clinical Sciences at Monash Health, Australia; ⁷Faculty of Medicine, Nursing & Health Sciences; Monash University, Australia; ⁸University of Melbourne, Melbourne, Australia; ⁹Monash University Centre for Obesity Research and Education, Alfred Hospital, Melbourne; ¹⁰Cabrini Hospital, Melbourne, Victoria.

Running head: Improved engraftment of esophageal PDTXs

Synopsis: This study reports the development and validation of a novel intramuscular transplantation technique that improves the engraftment rate of esophageal cancer patient-derived tumor xenografts.

Key Words: Patient derived xenograft (PDX), intramuscular (IM), cryopreservation, esophageal adenocarcinoma (EAC), esophageal squamous cell carcinoma (ESCC), preclinical model.

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Corresponding Author: Wayne A. Phillips PhD
Surgical Oncology Research Laboratory
Peter MacCallum Cancer Centre
Locked Bag 1 A'Beckett Street
Melbourne, VIC, 8006. Australia.
Email: wayne.phillips@petermac.org

Abstract

Background: Recently, there has been an increase in the availability of targeted molecular therapies for cancer treatment. However, the application of these approaches to esophageal cancer has been hampered by the relative lack of appropriate models for preclinical testing. Patient-derived tumor xenograft (PDTX) models are gaining popularity in many cancers. Unfortunately, these models have proven difficult to generate in esophageal cancer.

Aim: The purpose of this study was to improve the engraftment efficiency of esophageal PDTX.

Methods: Fresh pieces of esophageal tumors, obtained from either endoscopic biopsy or resected specimen, were transplanted into immunocompromised mice, either subcutaneously or intramuscularly and monitored for tumor formation.

Results: Using our novel intramuscular technique, successful engraftment was achieved from all 18 patients attempted. Thirteen of these PDTXs recapitulated the original patient tumors with respect to degree of differentiation, molecular and genetic profile, and chemotherapeutic response. However, lymphomatous transformation was observed in the remaining five PDTX. Successful engraftment was only achieved from one of six patients using the classical subcutaneous approach.

Discussion: Using our novel intramuscular transplant technique, we have achieved a much higher engraftment rate of PDTX when compared with other published studies. It is hoped that this advancement will help to expedite the development and testing of new therapies for esophageal cancer.

Background

Esophageal cancer is currently the eighth most common cancer worldwide and has an extremely high case fatality rate¹. Eighty three percent of all cases occur in developing countries, where squamous cell carcinoma is the main subtype². However, in the developed world, esophageal adenocarcinoma poses the greater problem, as its incidence has risen more than six-fold over the last four decades³. Currently, in the United States, only 17.5% of all patients who present with esophageal cancer will survive greater than five-years⁴. Alternative treatment strategies are needed to improve disease outcomes. Unfortunately, progress is being hampered by a lack of suitable pre-clinical models with which to study the disease and test new therapies.

One model that has shown great promise in the field of cancer research is patient-derived tumor xenografts (PDTX), in which patient tumor tissue is grown and expanded in immunocompromised mice. As these models recapitulate the original patient tumors with respect to histology, molecular and genetic profile and therapeutic response, they are being increasingly used as preclinical models for drug target validation and biomarker discovery⁵. PDTX also have the capacity to provide a perpetual tissue bank making them a valuable resource, particularly in esophageal cancer where the collection of treatment naïve tissue is often limited to small endoscopic biopsies obtained prior to neoadjuvant therapy⁶. Unfortunately, there has only been limited success in generating esophageal PDTX with success rates ranging between 30 to 38.5%⁷⁻¹³.

Given the importance of the PDX model to cancer research, refinements that lead to improvements in engraftment efficiencies are of great benefit¹⁴. One variable with the potential to improve engraftment efficiency is the transplantation site. Common sites for heterotopic transplantation include both subcutaneous (SC) and renal subcapsular spaces⁵. However, we hypothesized that an intramuscular (IM) transplantation site would be associated with an improved engraftment of PDX due to the well-vascularized transplant bed it provides.

Materials and Methods

Mice

Scid, NOD *scid*, NOD-*scid* IL2 receptor gamma chain knockout (NSG), and athymic nude mice were obtained from either the Animal Resource Centre (Canning Vale, Western Australia), the Walter & Eliza Hall Institute (Melbourne, Victoria) or bred internally at the Peter MacCallum Cancer Centre (Melbourne, Victoria). All mice were maintained and housed in the animal facility of the Peter MacCallum Cancer Centre. All experimental procedures involving animals were approved by the Peter MacCallum Cancer Centre Animal Experimental Ethics Committee and conducted in accordance with the National Health and Medical Research Council (NHMRC) *Australian code for the care and use of animals for scientific purposes* (8th edition, 2013).

Tissue Collection & Preparation

Patient tissue was collected from the Peter MacCallum Cancer Centre, the Cabrini Medical Centre, and other hospitals through the Victorian Cancer Biobank between March 2012 and June 2014. The collection and use of human tissue for this project was approved by the Ethics Committee of the Peter MacCallum Cancer Centre and carried out according to the NHMRC *National statement on ethical conduct in human research* (2007).

After obtaining informed consent, fresh samples of esophageal tumors were collected either by endoscopic biopsy or from surgical resection specimens and placed in chilled phosphate-buffered saline (PBS) supplemented with 200 U/ml penicillin and 200 µg/ml streptomycin (PBS⁺⁺) and kept on ice. Samples were divided into

approximately 2 mm³ sized pieces with any necrotic areas being discarded. Representative fragments were also snap frozen for DNA/RNA analysis or fixed in 10% buffered formalin for histology. The remaining tumor pieces were placed in MatrigelTM (BD Biosciences, NJ, USA) and kept on ice until implantation. All tissue was implanted within 90 minutes of collection.

Implantation & Monitoring

Mice were anesthetized via an intraperitoneal injection of 100 µl of anesthetic solution (10 mg/ml ketamine and 2 mg/ml xylazine) per 10 g of body weight and the dorsum of the mouse shaved and prepared with a 2% (v/v) chlorhexidine gluconate / 70% (v/v) isopropyl alcohol solution. Under aseptic conditions, a 15 mm midline incision was made, immediately caudal to the dorsal hump. Using blunt dissection, a skin flap was raised and the skin retracted laterally. For IM implantation, a superficial stay suture was placed within the dorsal musculature immediately caudal to the lowest rib using a 4/0 braided absorbable suture. After tenting the muscle fibers, an IM pocket was created using a combination of both sharp and blunt dissection until just large enough to accommodate a piece of tumor. A tumor piece coated in MatrigelTM was then placed in the IM pocket prior to it being sutured closed (Supplementary Fig. 1). For SC implantation, the tumor piece was implanted under the raised skin flap. Up to two separate transplantation sites were used per mouse. The skin was closed using a 3/0 braided absorbable suture. Mice were closely monitored for tumor formation and humanely euthanized once tumors reached the ethical limit of 1,500 mm³, as calculated by the formula $(\text{length} \times \text{width}^2)/2$, or at any sign of discomfort and/or stress.

Harvesting & Processing of PDTX

PDTX were carefully resected to ensure that the muscle layer was dissected free. After debridement of any necrotic areas, the PDTX was divided into 2 mm³ sized pieces and processed as per the original tissue. For cryopreservation, pieces were washed with PBS⁺⁺ then aliquoted into cryovials (up to 2 per cryovial) containing 1 ml of fetal calf serum containing 10% (v/v) dimethylsulfoxide (DMSO) and frozen to -80°C at approximately -1°C/min.

Histology & Immunohistochemistry

Sections of formalin-fixed paraffin-embedded (FFPE) tissue from all original patient derived tissues and PDTX were stained with haematoxylin and eosin (H&E). Immunohistochemistry was performed using antibodies against the following proteins: human mitochondrial antigen (MAB1273, Merck Millipore, Billerica, MA, 1:500), CK7 (OV-TL 12/30, DakoCytomation, Glostrup, Denmark, 1:50), CK19 (RCK108, DakoCytomation, 1:100).

DNA Extraction & Sequencing

DNA was extracted from freshly frozen pieces of tissue using the DNeasy Blood and Tissue kit (QIAGEN) as per the recommended protocol. Targeted sequencing across a panel of 48 known oncogenes was then performed using the Illumina TruSeq Amplicon Cancer Panel as previously described¹⁵.

Therapeutic Response

For assessment of chemotherapeutic response, PDTX were serially passaged into cohorts of up to 18 mice, using the IM technique. Athymic nude mice were selected for this purpose due to the ease of tumor detection and better tolerance of the chemotherapy. Once tumors reached a volume of 125 mm³, mice were randomized to receive either the matched patient chemotherapy (5-fluorouracil (5-FU) / cisplatin), or vehicle treatment. Cisplatin (DBLTM) was suspended in 0.9% (w/v) NaCl and administered at a dose of 4 mg/kg via intraperitoneal injection on a weekly basis for three weeks. 5-FU (DBLTM) was suspended in 0.9% NaCl and administered at 10 mg/kg via intraperitoneal injection on a daily basis for five days followed by nine rest days. This cycle was repeated twice. Vehicle consisted of 0.9% NaCl.

Results

Tumor samples from 23 patients were harvested from the esophagus, gastro-esophageal junction (GEJ) or peritoneum (metastatic deposit from a primary GEJ tumor) and included both adenocarcinoma and SCC. Between one and four tumor pieces (mode=2) from each patient were implanted. Samples from four patients were lost owing to the premature death of the host mice within the first month (i.e. before a tumor could establish) and were excluded from further analysis. Of the remaining patients, samples implanted using the IM technique had a per implant success rate of 24 from 37 (65%) and resulted in the successful engraftment of at least one implant from all 18 patients attempted with this method (Table 1). In comparison, the SC technique had a per implant success rate of only 1 of 11 (9%) and was associated with the successful engraftment of just 1 of 6 (16%) patients attempted with this method (Table 1).

All engrafted tissues were subjected to thorough histopathological analysis by an experienced histopathologist. All were demonstrated to be composed of human tissue, as demonstrated by staining with anti-human mitochondrial antibody (Fig. 1a). Of the 18 engrafted lines, 13 were confirmed to be of epithelial origin and to have maintained the same degree of differentiation (Fig. 1a) and expression of molecular markers (Fig. 1b) as their parent tumor. Thus we were able to successfully establish bona fide esophageal PDTX from 13 of 18 patients (72%) using the IM technique compared to just 1 from 6 patients by the SC method. Of the 13 esophageal PDTX lines successfully established, nine were obtained by endoscopic biopsy and four were

from surgical resection. Two of the 13 esophageal PDX were derived from patients who had been treated with neoadjuvant therapy.

Of the five IM xenografts that failed to recapitulate their parent tumors, four were confirmed to be B cell lymphomas and one was composed of a combination of both epithelial and lymphoid cells (Supplementary Fig. 2a&b). These lymphomas stained strongly for Epstein-Barr Virus (EBV) RNA (Supplementary Fig. 2d), indicating that latent EBV may be the driver of this lymphomatous transformation. These five lymphoma lines were also reproducibly metastatic in the mouse, with extranodal dissemination frequently noted in the lung, liver and spleen (data not shown).

All the validated esophageal PDX lines have now been passaged multiple times (up to seven) and continue to maintain their molecular and histological phenotypes. We have also been able to successfully re-establish four out of five esophageal PDX lines following cryopreservation. All passages were performed in NSG mice except for the failed cryopreserved sample, which was implanted into NOD SCID mice (Table 1).

Targeted sequencing of 48 known cancer related genes revealed the model to be relatively genetically stable. From a single PDX line (Patient 1), the original tumor was compared to seven PDX across three consecutive generations. Our results identified the presence of synonymous variants in five genes (FGFR3, PDGFRA, EGFR, RET, PTEN) and non-synonymous variants in three genes (HNF1A, TP53, STK11) in the parent tumor and all were maintained in the original PDX and subsequent passages, with the exception of one-second generation PDX in which the STK11 variant was not detected.

Importantly, we have also confirmed that the therapeutic responses observed in the patients are reproduced within the PDX. For example, patient 11, who had a poorly differentiated SCC of the mid to lower esophagus, experienced a complete response to the combination of chemotherapy (5-FU and cisplatin) and radiotherapy (50.4 Gy over 28 fractions). This complete response was mirrored in the matching PDX (Fig 2a). Similarly, patient 15, who was receiving treatment for a moderately differentiated adenocarcinoma of the GEJ, demonstrated only a partial response to the combination of chemotherapy (5-FU and cisplatin) and radiotherapy (50.4 Gy over 28 fractions) and this partial response was mirrored in the matching PDX (Fig. 2b).

Discussion

We report here a methodology for the improved establishment of PDTX from esophageal tumors. Using this novel IM technique we have been able to establish esophageal PDTX lines from 13 of 18 patients. These results suggest that this IM transplantation technique is more robust than the standard SC approach for the generation of esophageal PDTX. Given both our early success using the IM technique and the importance placed on generating additional PDTX lines from the limited quantity of tissue available, the majority of specimens within our series were implanted using the IM technique. For this reason, the numbers were too low for a direct comparison of the take rate between the IM and SC techniques. Nevertheless, when compared to the published engraftment rates across a range of reported esophageal PDTX series⁷⁻¹³, the IM technique appears to be superior. However, caution is needed when making such a comparison, as the small numbers mean it is not possible to standardize for variables such as ischemic time and tumor viability.

We postulate that the improved take rate observed with the IM technique can be attributed to the greater blood supply within the transplant bed. Evidence for this can be extrapolated from a study into the ectopic transplantation of ovarian tissue¹⁶. In this study, a comparison of the SC and IM transplantation sites was performed through a series of experiments involving the xenografting of normal rat ovarian tissue into nude mice. Results demonstrated that the SC transplantation site was associated with pericyte loss and tissue damage, whereas the IM site was associated with vascular maintenance and tissue preservation¹⁶. It is also well established that muscle tissue has greater autoregulatory control over blood flow compared to

cutaneous tissues¹⁷. This ensures that the temperature within the transplant bed remains relatively constant, protecting the tumor graft against hypothermic insult.

We have confirmed that the esophageal PDTX within our series are composed of human tissue and that they are representative of the original patient tumor with respect to degree of differentiation and both molecular and genetic phenotype. By assessing the response to standard chemotherapy, we have also produced evidence that the biology of the PDTX matches that of the original tumor. This highlights the potential power of well-validated PDTX as pre-clinical tools.

The feasibility of cryopreserving esophageal PDTX has also been demonstrated in this study. This has the potential to lead to significant reductions in the resources required to maintain perpetual PDTX bio-banks. It also gives researchers the ability to more readily share PDTX resources with collaborators.

Researchers also need to be cognizant of potential limitations when using PDTX models. Five of the initial 18 established xenografts (26%) underwent lymphomatous transformation. As it is not easy to distinguish poorly differentiated carcinomas from lymphomas by histology alone, it is important that poorly differentiated PDTX are tested for expression of lymphoma markers. Indeed, the process of lymphomatous transformation within xenografts has only recently been reported^{7,18,19}, but can be as high as 68% as reported in one series¹⁹. It is most likely that the lymphomas arise through transformation of EBV infected B-cells that have been transplanted with the tissue piece into a permissive environment and is akin to the development of lymphomas in immunodeficient patients, putatively modeling an EBV-driven post-

transplant lymphoproliferative disorder²⁰. Given the high rate of EBV infection within the general population it seems likely that this phenomenon has either been under-recognized or under-reported in the literature.

In summary, we have demonstrated that an IM transplant technique is associated with the improved engraftment of esophageal PDTX. Given the importance of the PDTX as a pre-clinical tool, it is hoped that such a refinement will lead to a greater understanding of cancer biology and improved therapeutics.

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Figure Legends

Figure 1: PDTX recapitulate the original patient tumor. (a) H&E staining (original tumor and PDTX) and immunohistochemistry using an anti-human mitochondrial antibody (PDTX only) in a poorly differentiated esophageal adenocarcinoma (Patient 1, top panels) and an esophageal SCC (Patient 11, bottom panels). (b) H&E staining and immunohistochemistry using CK7 and CK19 antibodies in a gastro-esophageal junctional (GEJ) adenocarcinoma (Patient 15, top panels) and the corresponding PDTX (bottom panels).

Figure 2: Assessment of tumor biology. Pre- and post-treatment ^{18}F -FDG-PET images and the growth curves for the respective PDTX with and without treatment with matched chemotherapy (cisplatin/5-FU) from representative patients who had either a complete metabolic response (a, Patient 11) or partial metabolic response (b, Patient 15) to their neoadjuvant treatment (cisplatin/5-FU). Shown are mean $\pm$ SEM. An unpaired t-test was performed on all data points using GraphPad Prism 5.0. * $p < 0.05$ for drug treated compared to vehicle control at same time point. Arrows indicate the site of the primary tumors.

Supplementary Figure Legends

Supplementary Figure 1: Demonstration of the intramuscular implantation technique and PDTX formation. (a) After the placement of a stay suture, a small IM pocket is created on the dorsum of the mouse, just large enough to accommodate the tumor piece. (b) Post implantation, the IM pocket is sutured closed with the implant evident immediately below the outer muscle fibers. (c) Gross view of an IM PDTX at time of harvest.

Supplementary Figure 2: Identification and characterization of lymphomagenic transformation within a subset of established PDTX. Immunohistochemistry using pan-cytokeratin AE1/AE3 (NCL-L-AE1/AE3, Novocastra/Leica Microsystems, Newcastle, UK, 1:100), CD20 (M7055, DakoCytomation, 1:200) and CD45 (M0701, DakoCytomation, 1:200) antibodies on original patient tumor and corresponding PDTX. The PDTX of patient 9 (a) is negative for the pan-cytokeratin marker, AE1/AE3, but positive for the common leukocyte antigen CD45 and the B-lymphocyte restricted antigen CD20, indicating that it is predominately composed of human B-cells. In comparison, the PDTX of patient 16 (b) exhibits a combination of both epithelial cells (nests of AE1/AE3 positive cells) and lymphocytes. (c) Further analysis via flow cytometry (performed as previously described²¹) of single cells derived from PDTX 14 confirmed that the lymphoid cells were CD19⁺ B cells, as demonstrated in the left plot. The abnormal B cell population was then gated and is depicted on the FSC vs SSC plot in blue (right plot), indicating that these cells are also large. (d) In situ hybridization for Epstein Barr Virus (blue staining) was performed on sections of FFPE PDTX using the INFORM EBER Probe and ISH iVIEW Blue Detection Kit (Ventana/Roche, Tucson, AZ) as per the manufacturer's

protocol and counterstained with eosin (pink). Left image is an EBV positive nasopharyngeal carcinoma (positive control); right image is a section from PDTX 9.

Table 1: The matched pathological features and engraftment results from implanted PDTX.

ID	Site ^a	Type	Grade	Tissue source	Therapy prior to implantation		Technique ^{b,c}		Mouse strain	Engraftment post cryopreservation ^b
					CT	RT	IM	SC		
1	GEJ	AC	Poor	Resection	Y	N	1/4	0/2	SCID	2/2
2	GEJ	AC	Poor	Resection	Y	Y	1/2		SCID	2/2
3	GEJ	AC	Mod	Resection	Y	N		0/4	NOD SCID	
4	Esophageal	AC & NE	Mod	Biopsy	N	N	2/2		NSG	
5	Esophageal	AC	Mod to Poor	Biopsy	N	N	1/2		NOD SCID	0/1
6	Esophageal	AC	In-situ	Biopsy	N	N	1/2	0/1	NSG	
7	Esophageal	SCC	Mod	Biopsy	N	N	1/1		NSG	
8	Esophageal	AC	Mod	Biopsy	N	N	1/1		NSG	
9	Esophageal	AC	Mod	Biopsy	N	N	1/1 ^d	0/1	NSG	
10	Esophageal	AC	Mod	Biopsy	N	N	2/2	1/2	NSG	1/1
11	Esophageal	SCC	Poor	Biopsy	N	N	2/2		NSG	1/1
12	Esophageal	SCC	Mod	Resection	N	N	1/1		NSG	
13	PM from GEJ	AC	Mod to Poor	Resection	N	N	1/2		NSG	
14	Esophageal	AC	Poor	Resection	Y	N	1/3		NSG	
15	GEJ	AC	Mod	Biopsy	N	N	2/3		NSG	
16	Esophageal	AC	Poor	Resection	N	N	1/2 ^e		NSG	
17	Esophageal	SCC	In-situ	Biopsy	N	N	1/1	0/1	NSG	
18	Esophageal	AC	Mod	Biopsy	N	N	1/2		NSG	
19	Esophageal	AC & NE	Poor	Biopsy	Y	N	3/4		NSG	

^a Abbreviations: GEJ, gastro-esophageal junction; PM, peritoneal metastasis; AC, adenocarcinoma; NE, neuroendocrine differentiation; SCC, squamous cell carcinoma; CT, chemotherapy; RT, radiotherapy; IM, intramuscular; SC, subcutaneous.

^b Fractions indicate the number of successful engraftments over the total number of implants.

^c Shaded, lymphomatous transformation within engrafted tumor; blank, not attempted.

^d Transformation occurred at second passage.

^e Mixed epithelial component.

Figure 1

Figure 2

Supplementary Figure 1

Supplementary Figure 2

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Author/s:

Read, M;Liu, D;Duong, CP;Cullinane, C;Murray, WK;Fennell, CM;Shortt, J;Westerman, D;Burton, P;Clemons, NJ;Phillips, WA

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