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Development of orthotopic tumour models using ultrasound-guided intrahepatic injection

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Mouse models of human diseases are an essential part of the translational pipeline. Orthotopic tumour mouse models are increasingly being used in cancer research due to their increased clinical relevance over subcutaneous xenograft models, particularly in relation to metastatic disease. In this study, we have developed orthotopic colorectal cancer liver metastases (CRCLM) and primary cholangiocarcinoma (CCA) models in BALB/c nude mice using minimally invasive ultrasound-guided intrahepatic injection. Due to its minimally invasive nature, the method reduced risk from surgical complications whilst being fast and easy to perform and resulted in measurable tumour volumes 1 to 3 weeks post-injection. Tumour volumes were monitored in vivo by weekly high-frequency ultrasound (HF-US) and/or twice weekly bioluminescence imaging (BLI) and confirmed with end-point histology. Take rates were high for human CRC cells (>73%) and for CCA cells (90%). We have demonstrated that this method reliably induces CRCLM and CCAs, in which tumour volume can be monitored throughout using HF-US and/or BLI. This provides a promising experimental tool for future testing of cancer therapeutics in an orthotopic model.

Metastatic cancer is the leading cause of death for patients diagnosed with colorectal cancer (CRC) with a five-year survival of stage-IV disease at diagnosis just 7–8%1. The most common site of metastasis is the liver, amounting to 70% of all cases2. Cholangiocarcinoma (CCA) although relatively rare, has a five-year survival of approximately 5%3. Both are characterised as being difficult to treat with poor long-term survival of patients. Therefore, there is a pressing need for more reliable models of these diseases for pre-clinical evaluation of anti-cancer therapies. Currently, orthotopic models recapitulating these human cancers are time-consuming to establish with poor or unreliable tumour formation.

In CRC, cancer cells from the primary tumour disseminate via the haematogenous route to secondary sites in the liver4,5. Mouse models of CRC and/or liver metastases are either transplantable or genetically modified, with the latter generally taking a long time to establish and rarely metastasises6,7. Transplantable models are much faster to generate but involve direct surgical implantation of cancer cells or tissues into the caecum or spleen, or direct injection into the portal vein8–11. The most commonly exploited models of colorectal cancer liver metastases (CRCLM) are injection site-dependant, either by surgical inoculation of the spleen or caecum involving implantation or subserosal injection12,13. Intrasplicenic injection results in the rapid formation of liver tumours by drainage into the hepatic portal vein via the splenic vein but often involves the removal of the spleen; a vital immune organ. Caecal injections are technically more challenging with a higher risk of post-surgical complications, but with the advantage of allowing the study of invasion from the primary site. However, this results in the slow (up to four months) and unreliable formation of metastases in the liver in addition to lung, lymph nodes and peritoneal tumours10.

Pre-clinical orthotopic models of CCA generally involve chemotoxic agents or gene knock-out techniques14,15. The process of generating these models is generally long and expensive. A syngeneic rat model of intrahepatic CCA using a rat-derived CCA cell line surgically implanted into the left hepatic duct with/surgical ligation of the bile duct, reported 100% success rates16. Although this model has the advantage of producing bile duct obstruction (which is a common feature of CCA), the development of the model involved invasive surgery and does not utilise human-derived cancer cells.

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The tumour microenvironment is thought to play an important role in influencing the growth, response to treatment and tumour immune responses in vivo. Tumours grown subcutaneously do not accurately represent the natural tumour microenvironment which contains fibroblasts, immune cells and an extracellular matrix which have all been shown to be influencing factors for tumour establishment and growth. Subcutaneous models, in general, have been poor predictors of drug efficacy in humans and therefore the need for more predictive models of human cancers. Hence, the generation of in vivo models of advanced disease in an orthotopic setting are necessary for more effective translation of novel therapies in order to improve treatment outcomes. Immune compromised or competent mice can be used depending on the cell lines used (patient-derived and/or human xenografts or syngeneic grafts) for testing of cytotoxic or immunotherapies, respectively.

To this end, we have developed a minimally-invasive method to create orthotopic liver tumours that recapitulate cancers in the liver. Ultrasound-guided percutaneous injections were used to develop a pre-clinical orthotopic model of CRCLM, which was minimally-invasive, fast and easy to perform and was amenable to monitoring tumour growth regularly and economically via HF-US. The human colorectal adenocarcinoma cell line, SW620, that was first derived from a lymph node metastasis was used with or without Matrigel in BALB/c nude mice to assess the feasibility of generating CRCLM. TFK-1, an extra-hepatic CCA cell line transfected to express luciferase (TFKLuc2B2), was used for the generation of an orthotopic CCA model which had the advantage of longitudinal tumour growth monitoring using BLI as well as HF-US imaging.

**Results**

**Development of an orthotopic model of CRCLM.** An orthotopic human CRCLM mouse model was developed using ten 5-week-old female BALB/c nude mice. Mice were randomised into two groups of five and anaesthetised with 5% (v/v) isoflurane gas in medical air at a flow rate of 2 L/min via an induction chamber, then positioned supine on the ultrasound platform with a nose cone for and maintained at 3% (v/v). Ultrasound gel was applied liberally to the abdomen and mice were imaged in B mode (2D) using a 40 MHz (RM-704) transducer with the VisualSonics Vevo 770 system, to determine and visualise the largest liver lobe (Fig. 1a). A 1 ml Terumo Myjector 29-gauge insulin syringe with a fixed 1.27 cm needle (Terumo, Tokyo, Japan) was aligned and advanced into the liver by freehand using the ultrasound monitor for reference. A 40 µl bolus of 20,000 SW620 CRC cells suspended in either PBS:Matrigel (1:1 v/v) or PBS alone was injected directly into the liver at a depth of 3 mm.
sis with poor glandular differentiation and mainly formed sheets of epithelial cells, typical of SW620 tumours.

Orthotopic tumours characteristically had necrotic islands and areas of geographic necrosis, which were analysed by H&E staining of paraffin-embedded fixed tissue and revealed single tumours with distinct boundaries. Tumour cells at the injection site by the formation of a plug and prevent leakage as seen where HF-US imaging missed one. Independent validation of the CRCLM model. This ultrasound-guided injection technique was independently validated by a second ultrasound operator using 15 5 to 7-week-old male BALB/c nude mice using Matrigel with the injection. All mice survived inoculation with weight monitoring and general health showing the procedure was well tolerated throughout. Animals were euthanised 47 days post-inoculation. This second cohort resulted in a take rate of 73% (11/15). Weekly HF-US imaging was used for longitudinal monitoring of tumour growth (Fig. 3). Mean (±SEM) tumour volume of 119.2 (±25.3) mm³ was determined at end-point, with a mean tumour doubling time of 4.5 days.

Development of an orthotopic model of primary cholangiocarcinoma (CCA). With the success and ease of this method for generation of CRCLM, the same technique was implemented to produce an orthotopic model of primary CCA. Ten 6 to 10-week-old female BALB/c nude mice were injected as described above with a 50 µl bolus of 5 million TFKLuc2B2 cells in PBS using HF-US (Matrigel not used). The gallbladder was used as a landmark for ultrasound-guided percutaneous injection into the liver as it was the closest relevant structure to the bile duct which could be located using ultrasound (Fig. 4a and Online resource 2). Animals were euthanised 19 days post-inoculation, and at end-point tumour take rate was 90% (9/10), two animals also had discrete intra-peritoneal tumours and one of these also had a subcutaneous tumour at the injection site (Online Resource 3). HF-US imaging was performed weekly and the median (± range) tumour volume was determined.
as 21.2 (9.0–32.5) mm³ 14 days post-injection (n = 6). Figure 4b shows a representative HF-US 3D tumour reconstruction image with the gallbladder nearby. At end-point, mice had tumours in the liver in close proximity to the gallbladder (Fig. 4c) and histological evaluation confirmed the presence of intrahepatic tumours close (<2 mm) to the gallbladder (Fig. 4d,e). One mouse was sacrificed at day 16 due to weight loss and abnormal behaviour. An orthotopic tumour was evident at necropsy with no obvious metastatic burden or cause of sudden deterioration.

In addition to HF-US imaging, bioluminescence imaging (BLI) was also performed up to twice weekly (Fig. 5), demonstrating an additional imaging modality for longitudinal monitoring of tumour burden in this model.

Discussion
To the best of our knowledge, this is the first known demonstration of non-surgical, orthotopic models of human CRCLM and primary CCA using ultrasound-guided intrahepatic injection. This method was minimally invasive thereby reducing risk from surgical complications, whilst fast and easy to perform resulting in measurable tumour volumes 1–3 weeks later. No loss in body mass or changes to normal behaviour were noted at any point during the experiments, with the exception of one CCA mouse at day-16. The use of a 29-gauge needle to inject...
cells resulted in no visible wound, an advantage over surgical inoculation which would require stitches, resulting in less inflammation and minimal healing time with this method. It has been widely suggested that pain and resulting stress can impact experimental outcomes resulting in poor reliability and reproducibility of experiments involving animals. This model is therefore, an excellent example of the National Centre for the Replacement, Refinement and Reduction of Animals in Research principles involving refinement of technique and development of techniques which minimise the 'pain, suffering, distress or lasting harm', directly improving welfare of animals in research. This model represents a refinement of current technique with fewer animals required than with traditional orthotopic models of CRC, as tumour take rates were high and subsequent growth could be monitored longitudinally and non-invasively.

Tumour burden consisted of obvious, large tumours at/near the injection site, with minimal dissemination into the abdominal cavity, a distinct advantage for interventional studies. The necessity of Matrigel may be cell line dependent, it was not necessary for tumour growth and was not used for the CCA cell line TFKLuc2B2, but was however shown to improve SW620 orthotopic rates from 40% to 73–100%. Matrigel is comprised of extracellular matrix proteins, collagen IV and growth factors which can be used to improve cell engraftment in difficult to grow cell lines or primary patient-derived cancer cells. Due to the small volumes that could be injected into the intrahepatic space, reduced cell numbers could be used (compared to subcutaneous tumour models) and the Matrigel may have provided a more favourable environment for growth. The Matrigel may also have held the cells in place by the formation of a plug (Matrigel solidifies rapidly above 10°C) therefore preventing leakage of cells from the injection site, as seen in the group without Matrigel. Recently, a group has successfully demonstrated surgical intrahepatic inoculation of hepatocellular carcinoma cells with 100% take rates, however, 50% of these

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Tumour volume (mm³)</th>
<th>With Matrigel</th>
<th>Without Matrigel</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>93.2</td>
<td>81.6</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>81.4</td>
<td>N/O</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>54.1</td>
<td>N/T</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>30.3</td>
<td>N/T</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>N/R</td>
<td>N/T</td>
<td></td>
</tr>
<tr>
<td>Take rate</td>
<td>4/4 (100%)</td>
<td>2/5 (40%)</td>
<td></td>
</tr>
<tr>
<td>Median volume (mm³)</td>
<td>67.7</td>
<td>81.56</td>
<td></td>
</tr>
<tr>
<td>Median doubling time (days)</td>
<td>3.9</td>
<td>3.4</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. 3D volume measurements of orthotopic CRC tumours by HF-US, take rates and tumour doubling time. Take rates and final tumour volumes 35 days after tumour inoculation. N/R = non-recovery from anaesthesia, N/O = tumour not observed by US due to low position, N/T = no tumours. Median value given where possible.

Figure 3. Orthotopic CRCLM tumour growth curve. This technique was independently validated by a second ultrasound operator using Matrigel with ultrasound-guided intrahepatic injection of SW620s and resulted in a take rate of 73% (11/15). Weekly HF-US imaging was used for longitudinal monitoring of tumour growth. At 47 days post-inoculation (end-point), tumour volume was determined as 119.2 ± 25.3 mm³ (mean ± SEM) with a mean tumour doubling time of 4.5 days.
also showed abdominal and pelvic tumours from leakage during intrahepatic injection or potentially a cell line specific result28. The non-surgical transplantation of CRC cells into liver has not been done before, but orthotopic pancreatic and bladder cancer models have been successfully developed using ultrasound-guided injection, demonstrating the diversity of models which can be created using minimally invasive methods29,30.

HF-US and BLI were used to identify the location of tumours and to monitor the progress of tumour growth in vivo. Both are relatively cheap, quick and safe imaging modalities as demonstrated here. Without the use of imaging, the outcome may not be known until end-point dissection and histological analysis, which would be time-consuming and require many more mice to determine appropriate end-points. As shown here, end-point liver mass can also be used to quantitate the degree of CRCLM tumour growth, as livers with tumours increased in mass significantly compared to those without. Other imaging modalities such as BLI (as shown here), magnetic resonance imaging, micro computed tomography or fluorescence31–33 may also be used for in vivo tumour volume assessment but may not be as economical, rapid or as accurate as HF-US for measurement of tumour volumes in vivo34.

Orthotopic tumours grow differently to the commonly used subcutaneous models and therefore the importance of using a model which takes into consideration the cancers natural microenvironment is vital for faster and more reliable clinical translation. The formation of large single tumours could reflect late-stage CRC, where large tumours in the liver would require de-bulking (i.e. obstructing portal vein) via adjuvant chemotherapy or radiation, prior to surgical resection. This model provides a promising experimental tool for future testing of anti-cancer agents for the management of metastatic CRC or primary CCA with the possibility of primary liver tumours such as hepatocellular carcinoma, hepatoblastoma or liver metastases from other cancers. The use of syngeneic cell lines in immunocompetent mice or patient-derived cells in immunodeficient mice should also be possible using this method and requires further evaluation.

In summary, this method has successfully demonstrated the development of a minimally-invasive model of CRCLM and CCA. The relatively quick and reliable formation of tumours allows for ease of monitoring of tumour volume via HF-US or BLI, and therefore a means of testing efficacy of novel therapeutics quickly and easily. This model provides a promising experimental tool for future testing of anti-cancer agents in an orthotopic setting.

**Figure 4.** Representative images of HF-US imaging and subsequent orthotopic CCA tumours. (a) HF-US image of mouse liver prior to inoculation. The gallbladder (GB) was used as a landmark for injection. Portal vein (PV) also shown. (b) 3D reconstruction (matrix) of orthotopic tumour volume (day 14 post-inoculation) with gallbladder (arrow). 3D scans of tumours were performed using HF-US using a 40 MHz (RM-704) transducer on a motorised arm, with the VisualSonics Vevo 770 system. The 3D reconstructions were generated post-acquisition using the Vevo 770 version 3 software. (c) Representative mouse liver at end-point (19 days). Tumour (T) in close proximity to gallbladder (GB) indicated. (d,e) Liver (L) sections of two orthotopic tumours stained with H&E to demonstrate proximity of orthotopic tumour (<2 mm) to gallbladder (GB).
Methods

Cell culture. The SW620 cell line was originally obtained from the American Type Culture Collection (ATCC). Human extrahepatic CCA cells TFK-1 (originally derived from mid-bile duct tumour35) were transfected with luciferase for bioluminescence imaging, clones were selected and one was termed TFKLuc2B2. Cells were regularly screened for mycoplasma and were short random repeat (STR) DNA profile authenticated. Cells were maintained in vented T150 tissue culture flasks (Corning Life Sciences, UK) using Roswell Park Memorial Institute medium 1640 (RPMI Medium 1640, Gibco™) supplemented with 10% (v/v) foetal calf serum (FCS) (Invitrogen, UK) at 37°C in a 5% CO₂ atmosphere. Cells were passed at 70–80% confluence by washing with sterile phosphate buffered saline (PBS) pH 7.4 (Invitrogen, UK) before being harvested by trypsinisation. Culture medium was added back into the flask to block the action of trypsin before cells were collected and centrifuged at 400 g for 5 minutes using an Eppendorf™ 5810 R centrifuge (Eppendorf™ Hamburg, Germany). Cells were re-suspended in PBS and the total cell count was obtained manually using a Neubauer haemocytometer, and the concentration adjusted using PBS. SW620 cells were further adjusted to 5 × 10⁵ cells/ml using ice-cold Matrigel (Geltrex™ LDEV-free reduced growth factor basement membrane matrix without phenol red, Life Technologies) or PBS immediately prior to injection. TFKLuc2B2 cells were adjusted to 1 × 10⁸ cells/ml using PBS prior to injection.

Animals. BALB/c nude mice were originally obtained from Charles River Laboratories (Kent, UK) and maintained in-house. All mice were maintained under high health status conditions and were specific pathogen free status. Animals were housed in individually ventilated cages with access to food and water ad libitum. All procedures were approved by the UK Home Office and carried out according to the Animals (Scientific Procedures) Act 1986.

HF-US imaging. HF-US was performed weekly in the CRCLM model and at 3 weeks for the CCA model to measure tumour volume. Using a Vevo 770 HF-US system (Fujifilm VisualSonics, Inc, Ontario, Canada) a 40 MHz (RM-704) transducer (VisualSonics, Inc, Ontario, Canada) was held above the animal in supine position36, and imaged in B mode. Vevo 770 version 3 software (Fujifilm VisualSonics, Inc, Ontario, Canada) was used for post-acquisition 3D reconstructions and used to calculate tumour volumes37. A video showing HF-US imaging of a CRCLM tumour is included in the Online Resource 4.

Bioluminescence imaging (BLI). For in vivo BLI of CCA tumours, 150 mg/kg of D-Luciferin (Promega, Madison, WI, USA) was administered intraperitoneally five minutes prior to imaging using an IVIS Spectrum (PerkinElmer, Inc. Waltham, MA, USA). Auto-exposure settings were used with a maximum exposure time of
one minute. Living image software version 4.4 (PerkinElmer, Inc. Waltham, MA, USA) was used to analyse bioluminescence signals. Standardised regions of interest were used to calculate average radiance (photons/second/cm²/steradian) across imaging sessions.

**Histology.** Immediately after sacrifice by cervical dislocation, livers were collected and washed in ice-cold PBS, weighed and fixed by immersion in 4% (w/v) paraformaldehyde. Tissues were blocked in paraffin wax, 5µm sections cut and stained with Haematoxylin and Eosin (H&E, Sigma, UK) for visual examination. Images were obtained using an Eclipse E1000 light microscope and NIS-Elements BR software (Nikon Instruments Inc., Tokyo, Japan).

**Immunohistochemistry.** CD31-positive blood vessels were stained following the manufacturer’s instructions. In brief, sections were deparaffinised and heat-mediated antigen retrieval in 10 mM citrate buffer (pH6) was performed for 10 minutes. Endogenous peroxidases were blocked with 0.3% v/v hydrogen peroxide followed by blocking of endogenous avidin/biotin (Vectorlabs, Burlingame, USA). Sections were then incubated for 1-hour with the primary antibody (1:20 rat anti-mouse CD31, clone SZ31, Dianova GmbH, Germany). After washing, a biotinylated anti-rat secondary antibody was applied for 30 minutes (1:200 Dako, UK). ABC/HRP solutions (ABC/HRP Complex Kit, Vectorlabs, Burlingame, USA) were then applied and visualised with 3,3’-Diaminobenzidine (DAB, Dako, UK). Slides were scanned using an Aperio slide scanner (Leica Biosystems, Wetzlar, Germany) at × 20 magnification. Ten 0.25 mm² boxes were placed randomly throughout the tumour section using RandomSpot software version 6.02. The number of CD31 positive vessel were counted manually. Microvessel density was calculated as the number of CD31 positive vessels per mm².

**Statistical analyses.** All statistical analyses were performed using GraphPad Prism 7 (©2017 GraphPad Software, Inc., CA, USA). The type of analysis used in each experiment and statistical significance are shown in figure legends.

**References**


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Author Contributions
L.E.M., I.W., N.I. and G.M. designed and performed experiments, collected and analysed data. L.E.M. and I.W. prepared figures and drafted the manuscript. A.F.M. and R.P. supervised the project. P.L.C. conceived, designed and supervised the project. All authors revised the manuscript and have approved the final version.

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