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SMALL ANIMAL IGRT SPECIAL FEATURE: FULL PAPER

Pre-clinical imaging for establishment and comparison of orthotopic non-small cell lung carcinoma: in search for models reflecting clinical scenarios

^{1,2,3}ROZINA AKTAR, MSc, ^{1,2,3}ANTJE DIETRICH, PhD, ^{1,4,5}FALK TILLNER, MSc, ^{1,2,3}SHADY KOTB, PhD, ^{1,2,3,4}STEFFEN LÖCK, PhD, ⁶HENNING WILLERS, MD, ^{1,2,3,4,5}MICHAEL BAUMANN, MD, PhD, ^{1,2,3,4,5,7}MECHTHILD KRAUSE, MD, PhD and ^{1,3,4,7}REBECCA BÜTOF, MD

¹OncoRay–National Center for Radiation Research in Oncology, Faculty of Medicine and University Hospital Carl Gustav Carus, Technische Universität Dresden, Helmholtz-Zentrum Dresden-Rossendorf, Dresden, Germany

²German Cancer Consortium (DKTK), Partner Site Dresden, Dresden, Germany

³German Cancer Research Center (DKFZ), Heidelberg, Germany

⁴Department of Radiotherapy and Radiation Oncology, Faculty of Medicine and University Hospital Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany

⁵Helmholtz-Zentrum Dresden-Rossendorf, Institute of Radiooncology–OncoRay, Dresden, Germany

⁶Department of Radiation Oncology, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA

⁷National Center for Tumor Diseases (NCT), Partner Site Dresden, Germany; German Cancer Research Center (DKFZ), Heidelberg, Germany; Faculty of Medicine and University Hospital Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany, and; Helmholtz Association/Helmholtz-Zentrum Dresden-Rossendorf (HZDR), Dresden, Germany

Address correspondence to: Dr Rebecca Bütof

E-mail: rebecca.buetof@uniklinikum-dresden.de

Objective: Clinically relevant animal models of non-small cell lung carcinoma (NSCLC) are required for the validation of novel treatments. We compared two different orthotopic transplantation techniques as well as imaging modalities to identify suitable mouse models mimicking clinical scenarios.

Methods: We used three genomically diverse NSCLC cell lines [National Cancer Institute (NCI)-H1703 adenocarcinoma, NCI-H23 adenocarcinoma and A549 adenocarcinoma] for implanting tumour cells either as spheroids or cell suspension into lung parenchyma. Bioluminescence imaging (BLI) and contrast-enhanced cone beam CT (CBCT) were performed twice weekly to monitor tumour growth. Tumour histological data and microenvironmental parameters were determined.

Results: Tumour development after spheroid-based transplantation differs probably due to the integrity of spheroids, as H1703 developed single localised nodules, whereas H23 showed diffuse metastatic spread starting early after transplantation. A549 transplantation as cell suspension with the help of a stereotactic system was associated with initial single localised tumour growth

and eventual metastatic spread. Imaging techniques were successfully applied to monitor longitudinal tumour growth: BLI revealed highly sensitive qualitative data, whereas CBCT was associated with less sensitive quantitative data. Histology revealed significant model-dependent heterogeneity in proliferation, hypoxia, perfusion and necrosis.

Conclusion: Our developed orthotopic NSCLC tumours have similarity with biological growth behaviour comparable to that seen in the clinic and could therefore be used as attractive models to study tumour biology and evaluate new therapeutic strategies. The use of human cancer cell lines facilitates testing of different genomic tumour profiles that may affect treatment outcomes.

Advances in knowledge: The combination of different imaging modalities to identify tumour growth with subsequent use in treatment planning and orthotopic transplantation techniques to develop initially single lesions to ultimate metastases pave the way towards representative pre-clinical NSCLC models for experimental testing of novel therapeutic options in future studies.

INTRODUCTION

Compared to all other tumour entities, lung cancer is estimated to cause the highest number of cancer-related deaths in USA.¹ Non-small cell lung carcinoma (NSCLC) is the

most common (~85%) group of lung tumours and approximately 70% of NSCLC patients are diagnosed in advanced stages with regional or distant metastases (Stage III and IV) resulting in poor 5-year overall survival rates of 10–15%.^{2,3}

One contributing factor for slow progress in treatment advances in NSCLC could be the lack of pre-clinical models that capture the clinical scenario and help to understand the mechanisms of disease progression as well as for testing therapeutic response in pre-clinical trials. Therefore, there is a necessity of developing reproducible and reliable, clinically relevant animal models of NSCLC to implement and validate therapeutic options in the era of personalised medicine.

In recent years, orthotopic animal models have been extensively reported and considered as better representatives of clinical circumstances by realistically mirroring the progression pattern of cancer in an appropriate microenvironment.⁴ Several tumour implantation methods have been previously employed to develop orthotopic NSCLC models, each with their pros and cons. Among them, intratracheal injection of cells in solution often leads to diffuse initial spreading of cells in both lungs.⁵ In contrast, transbronchial application of cell suspensions has the advantage of resulting in single orthotopic tumours in mice. However, the need for tracheostomies or endotracheal intubation with the help of a surgical microscope makes this method technically very challenging.^{6,7} Open percutaneous transplantation of cell suspension is potentially associated with pleural seeding during injection and every invasive surgical procedure could be associated with high perioperative mortality.⁸ Transpleural orthotopic injection is a promising method, which allows transplantation of a moderate volume of cell suspension through the intercostal space either using a conventional needle or with the help of a stereotactic system.^{8,9} To avoid rapid metastatic spread due to unwanted cell distribution in the injection channel, spheroid transplantation may be a method to optimise transpleural transplantation. A limitation here is that not all cell lines develop spheroids.¹⁰

A longitudinal study of orthotopic lung cancer development without euthanising the animals at different time points requires imaging modalities. Distinctive imaging techniques are optimised for sensitive and volumetric monitoring of murine experimental lung tumour growth as bioluminescence imaging (BLI), MRI and cone beam CT (CBCT).^{6,8,11–14} However, the lung remains a challenging organ to image due to its intrinsic properties; in particular its continuous respiratory motion. To the best of our knowledge, no systematic comparison of different imaging modalities has been published for the suitability of longitudinal monitoring of orthotopic lung cancer development.

The aim of our investigation was to compare spheroid and cell suspension transplantation techniques regarding reproducibility of human NSCLC progression from initially localised lesions to metastatic disease. In addition, we tried to define a suitable tumour size for the start of target-specific radiation treatment for pre-clinical radio-oncological trials. Furthermore, we performed contrast-enhanced CBCT integrated in our small animal image-guided radiation therapy platform^{15,16} and BLI in order to improve tumour monitoring to provide models mimicking early, mid and late clinical diagnosis.

In this work, we established orthotopic lung tumour models using three different NSCLC cell lines. We transplanted tumour spheroids (~600 µm diameter) transpleurally, as this was hypothesised to reduce the risk of spread in the injection track. As not all cell lines form spheroids, we additionally used a stereotactic transplantation technique, adapted from reference,⁹ to inject a relatively low amount of cell suspension (3–5 µl) precisely into the transplantation site. The imaging modalities were compared regarding their sensitivity, limit of detection and accuracy of tumour measurements. Concurrently, we investigated the tumour microenvironment (hypoxia, vessels, necrosis and perfusion) as a major factor of radioresistance with an objective of identifying tumour models that best resemble the clinical situation.

METHODS AND MATERIALS

Cell lines and cell culture

The human cell lines A549 (adenocarcinoma), National Cancer Institute (NCI)-H23 (adenocarcinoma) and NCI-H1703 (adenosquamous carcinoma) were purchased from American Type Culture Collection. Transduction of all three cell lines with firefly luciferase (pHATrick-T2A-Puro-Ff_Luciferase) was done in the laboratory of Professor Achim Temme, University Hospital Carl Gustav Carus, Dresden, Germany. A549 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Biochrom GmbH, Germany). NCI-H23 and NCI-H1703 cell lines were maintained in Roswell Park Memorial Institute medium (RPMI, Biochrom GmbH, Germany) containing 10% fetal calf serum (Sigma Aldrich, Germany) and 1% penicillin/streptomycin (Biochrom GmbH, Germany) at 37°C in 5% carbon dioxide.

Spheroids have been developed as previously described.¹⁰ Briefly, we established 600 µm diameter spheroids with different cell lines on day 4 after inoculation of distinct cell numbers in liquid overlay culture in 96-well plates.¹⁷ To examine spheroid integrity and size, each well was imaged with a phase contrast microscope (Axiovert200/M-based system, Zeiss, Germany) using a 10x objective on day 4 after spheroids initiation. AxioVision 4.8 software (Zeiss, Germany) was used to analyse spheroids through appropriate image processing algorithms.

To determine plating efficiency at 0 and 2 Gy, a defined number of cells for each cell line was seeded into T25 cell culture flasks. Three flasks for each cell line were irradiated with 2 Gy (~1 Gy min⁻¹). After 14 days colony formation was stopped and counted.

Animals and termination criteria

Animal experiments and animal facilities were in compliance with German animal welfare regulations and OncoRay institutional guidelines. Male and female NMRI (nu/nu) mice at the age of 7–14 weeks were used for the experiments and obtained from our pathogen-free animal breeding facility. The mice underwent total body irradiation 2–5 days prior to tumour transplantation with 4 Gy (200 kV, 1 Gy min⁻¹) to further reduce the residual immune system of the nude mice.

Transpleural injection of spheroids (NCI-H23, NCI-H1703) and cell suspension injection (A549) were done in an equal

number of mice ($n = 8$). Imaging was first performed 1 week after transplantation and twice weekly afterwards until reaching prespecified termination criteria (e.g. weight loss $\geq 20\%$, presence of enlarged regional lymph nodes, tumour volume or tumour diameter exceeding 0.12 cm^3 and 6 mm, respectively, pulmonary dysfunctions as altered respiratory rate and cyanosis, impairment of general condition).

Orthotopic transplantation

Injection of cell suspension

The surgical procedure was adapted as previously described⁹ by using a stereotactic system (Type 51730, Stoelting Co., Wood Dale, IL).

Briefly, cultured luciferase-expressing A549 cells (1×10^5 or 5×10^4 per 3–5 μl) were harvested and suspended in Phosphate Buffered Saline (PBS, Biochrom GmbH, Germany). The cells were mixed with Matrigel (Gibco, Gaithersburg, MD) at 1:1 ratio and kept on ice until transplantation. The cell mixture was gently mixed before injection and transferred into a 10 μl Hamilton syringe (Hamilton, Romania). Mice were anaesthetised by intraperitoneal injection (i.p.) of ketamine (100 mg kg^{-1}) (WDT, Germany) and xylazine (10 mg kg^{-1}) (Serumwerk Bernburg, Germany). The mice were then kept in the lateral position with the right chest facing up. A 0.5–1 cm long skin incision was made just below the scapula. Next, the chest wall muscles were incised to reach the intercostal space. Then the parietal pleura was identified and cut. The injection site was determined at the intercostal space between the fourth and fifth rib along the right posterior axillary line. The syringe was gradually and very slowly advanced to touch the intercostal space gently and penetrate up to 3.5 mm into the lung parenchyma over a time period of 2–3 min. After an incubation period of 1 min, the cell suspension was injected into the lung at a rate of $1 \mu\text{l min}^{-1}$. The syringe was kept in place for additional 2 min to prevent spilling back of cell suspension through the injection track. The syringe was withdrawn slowly and the covering muscles and the skin were closed with metallic clips (Becton Dickinson, NJ). Finally, mice were given subcutaneous Carprofen (Zoetis, Germany) for analgesia and kept in a preheated pad (37°C) until recovery.

Spheroid injection

Mice were anaesthetised and positioned according to the protocol above. A 0.5–1 cm long incision was made below the right scapula 0.5 cm left to the posterior axillary line. The spheroids ($\sim 600 \mu\text{m}$ diameter) were aspirated with a 100 μm pipettor and loaded into a 20G cannula, in the course of which PBS was initially introduced and then slightly aspirated so that the spheroid was inserted in the front of the cannula. This method was used to prevent disintegration of the spheroids through the syringe. The transplantation site was determined at the intercostal space between the fourth and fifth rib. The spheroids were injected 3.5 to 5 mm deep into the lung parenchyma. After 10 s, the needle was withdrawn slowly. Aftercare was done as described above.

Bioluminescence imaging

For H23 and H1703 cell lines BLI was performed with the IVIS Spectrum (PerkinElmer, Waltham, MA). Due to malfunction of

the system, BLI investigations for A549 cell line were performed with the *In-Vivo Xtreme II* (Bruker, UK). Both systems are comparable regarding image quality and sensitivity. Before imaging, D-luciferin (PerkinElmer, EU) was administered by i.p. injection at a dose of 100 mg/kg and the animals were anaesthetised by using inhalational anaesthesia (2.5% isoflurane, Baxter, Germany). Images were taken 15 min post D-luciferin injection at the plateau phase of signal intensity, which was determined separately for each cell line. The imaging parameters for all BLI acquisitions with the IVIS Spectrum were as follows: exposure time-auto, binning-M, 8 f/stop-f1 and field of view-12.5 cm and for *In-Vivo Xtreme II*: exposure time-60 s, binning- 2×2 , f/stop-1.1 and FOV-12 cm. The images were processed using the Living Image 4.3.1 software (PerkinElmer, Waltham, MA) and Bruker Molecular Imaging software (Bruker, UK), respectively. The pseudo colour image, which was an illustration of the spatial distribution of identified photons, was overlaid on a greyscale photographic image. A region of interest (ROI) was created around the BLI for quantification of signal intensity.

CBCT

In addition to BLI, tumour growth was monitored by contrast-enhanced CBCT. The mice were i.p. injected with 150 μl contrast agent Ultravist 370 (769 mg Iopromide - corresponding to 370 mg bound iodine, Bayer, Germany). The CBCT examinations were done 10 min post injection under inhalational anaesthesia as described above. Images were obtained at the SAIGRT platform^{15,16} for the whole mice with following parameters: 40 kV and 6 mA with 1.06 min exposure time. CBCT reconstruction was done using ConeBeam software¹⁵ and visualised as well as contoured by one observer using the in-house developed Geis-terr software.^{15,18}

Histology and staining

Pimonidazole (i.p., 0.1 mg g^{-1} body weight, dissolved in 10 mg ml^{-1} of 0.9% NaCl) and Hoechst 3,3342 (intravenously, 15 mg kg^{-1} body weight, dissolved in 0.05 ml PBS) were injected before euthanising the animals. Tumour samples with lungs were removed and cryopreserved in Optimal Cutting Temperature compound (OCT, A. Hartenstein GmbH, Germany). 10 μm thick sections were cut (CryoStar NX70, Thermo Scientific, MA) in each 100 μm step and stored for further staining.

Haematoxylin/Eosin staining was done to identify the presence of viable tumour cells. To identify proliferating cells of human origin, a human-specific Ki-67 antibody was used for immunohistochemistry. For epitope retrieval, sections were boiled in 90°C for 85 min in a microwave in 0.1 M citrate buffer, pH 7.2. Slides were incubated with the biotinylated primary Ki-67 antibody: monoclonal mouse anti human Ki-67 Antigen, clone MIB-1 (Dako, Germany), dilution 1:200. Diaminobenzidine (Dako, Germany) was used for colour development and counterstaining was done with hematoxylin. To investigate the tumour micromilieu, immunofluorescence staining of tumour vessels, pimonidazole positive areas (=hypoxia) and vessel perfusion was done.¹⁹ Sections were incubated overnight with the following primary antibodies: pimonidazole antibody, polyclonal, rabbit (HPI, CA), dilution 1:2500 and anti mouse CD31, rat, monoclonal, clone

Mec 13.3 (BD Biosciences, Germany), dilution 1:500. Anti rabbit FITC (Jackson ImmunoResearch, PA), dilution 1:300 and anti rat TRITC (Jackson ImmunoResearch, PA), dilution 1:150 were used as secondary antibodies, respectively. Proliferation indices (percentage of Ki-67 positive cells) were assessed in one representative tumour for each model in 10 randomly selected microscopic high-power fields (40x magnification). Slides containing histological sections of the tumour were imaged using AxioImager M1 microscope (Zeiss, Germany).

Statistical methods

We present data from tumour-bearing mice. Animals that developed initial subcutaneous tumours, did not show tumour growth or suffered early deaths have been censored.

Statistical analyses were performed with GraphPad Prism 6.03 software (GraphPad Software, La Jolla, CA). Analysis of variance was used to compare the percentage of Ki-67 positive cells between tumour models. Post-hoc comparison was performed using Bonferroni correction for multiple testing. Logarithmic values of tumour volumes obtained from CBCT at each time point were correlated with the corresponding logarithmic bioluminescence values using Pearson correlation. p -values < 0.05 were considered statistically significant and two-sided test were applied. In all graphs, the mean value and the corresponding standard error are displayed.

RESULTS

Overall, we observed take rates of 100% ($n = 8$) for NCI-H1703, 50% for NCI-H23 and 50% for A549 cells based on imaging and histological data, as described below.

The *in vitro* survival fraction at 2 Gy for all three cell lines was observed as follows: H1703: 0.57, H23:0.25 and A549:0.57.

Tumour growth assessment

Non-invasive longitudinal tumour monitoring was done using both BLI and CBCT. Qualitative (Figure 1a,e and h) and quantitative (Figure 1b–d, f–g and i–l) tumour progression was observed with both imaging modalities. Figure 1 shows contrast-enhanced CBCT and BLI signals from a representative tumour-bearing animal for each cell line. BLI values and CBCT tumour volumes were quantified at each time point and revealed a steady increase over time. BLI was shown to be more suitable to identify tumour development (NCI-H1703–100%, NCI-H23–50% and A549–87.5%) compared to CBCT, demonstrating sensitivity as well as reliability of this imaging technique. In the H23 cell line, tumour development was successfully monitored in BLI images, but corresponding CBCT images did not reveal consistent evidence of tumour development. However, atelectasis was shown at late time points (Figure 1e, CBCT). It has to be noted that A549 tumours, which were transplanted through cell suspension, showed extensive metastases in the same lung field (Figure 1h, CBCT) whereas H1703 tumours were localised (Figure 1a, CBCT) during the whole growth period.

Correlation of different imaging techniques

For the models H1703 and A549, logarithmic values of CBCT-based tumour volumes at each time point were compared with the corresponding logarithmic bioluminescence values (Figure 2). A significant correlation (Pearson correlation, $r = 0.64$, $p < 0.0001$) between optical imaging values and CBCT-based tumour volumes could be shown for A549 tumours (Figure 2a). For H1703 tumours the correlation was similar ($r = 0.55$) but due to the lower sample size not significantly different from 0 (Figure 2b). However, for both cell lines correlation value is not high enough to draw a final conclusion.

Histological/microenvironmental characteristics and growth fraction

Histological examination showed that the pattern of tumour formation varied according to the mode of cell implantation, *i.e.* cell suspension vs spheroids, and in the case of the latter also probably depended on the stability of spheroids. H1703 cells (spheroid transplantation) primarily formed single nodules adjacent to the lung parenchyma (Figure 3a, yellow arrow), whereas H23 cells (spheroid transplantation) produced multiple pulmonary and extra pulmonary nodules (Figure 3a, yellow arrows). However, H23 spheroids were found to be very loosely packed so that the early spread might have been a result of breakage of spheroids during transplantation. On the other hand, A549 tumour cells (stereotactic cell suspension transplantation) were observed primarily growing within pulmonary spaces but showing early metastatic spread with multiple nodules within the lung (Figure 3a, yellow arrows). Necrosis developed in all three models (Figure 3a, black arrows). In A549, 37.5% of all tumour-bearing mice developed subcutaneous metastases over time at the injection site (data not shown).

NSCLC orthotopic tumour sections were stained with anti human Ki-67 antibody to determine proliferation indices (Figure 3b). The mean number of Ki-67 positive nuclei differed significantly between the cell lines, with 59.17 ± 1.88 SEM for H1703, 50.67 ± 2.04 SEM for H23 and 19.53 ± 2.41 SEM for A549 tumours. The bar graph in Figure 3c shows the proliferation index of the different tumour models.

The tumour microenvironment varied between the different models (Figure 4). H1703 tumours showed more vascularity and less necrosis accompanied with less pimonidazole (hypoxia) positive areas (Figures 3a, 4a and c). In contrast, A549 tumours were associated with less vascularity, but more necrosis and hypoxia (Figures 3a, 4b and d). These findings correlated also with the proliferation index of the corresponding tumours, *e.g.* H1703 showed a high proliferation index (Figure 3c); vice versa was observed in A549.

DISCUSSION

Human NSCLC orthotopic xenografts have been investigated over the past two decades. These models are considered to better reflect anatomical location and microenvironmental characteristics of patient tumours. They also allow us to assess the therapeutic window of novel therapies for tumours growing in those tissues which are relevant in the clinical situation.

Figure 1. Growth of orthotopic NSCLC of three different cell lines (Upper-NCI-H1703, middle-NCI-H23 and lower-A549) assessed by BLI and CBCT. Images show tumour growth in a representative animal using both imaging modalities (a, e and h). BLI signal for each individual mouse (b, f, i) and mean BLI signal $\pm$ SEM (c, g, j) for all mice were plotted over time. The tumour volume acquired by CBCT for each mouse bearing a solid lesion ($n = 2$ for H1703 and $n = 4$ for A549) (d, k) and mean tumour volume $\pm$ SEM (l) are shown. No specific tumour signal was observed in CBCT images for H23 tumours but possible atelectasis (e, arrow in CBCT). BLI, bioluminescence; CBCT, cone beam CT; NCI, National Cancer Institute; NSCLC, non-small cell lung carcinoma.

Several transplantation techniques have been tested, including intrabronchial injection or intrapleural implantation into lung parenchyma after skin incision or surgical exploration of the pleura.^{20–24} The most suitable transplantation techniques and reliable models without major transplantation complications are still not established.

In this study, we used two different transplantation techniques to establish human lung cancer tumours orthotopically in NMRI

(nu/nu) nude mice. Immunodeficient mouse strains have the disadvantage of missing possibilities to investigate the role of the immune system for tumour growth and therapy response. With the help of genetically engineered mouse models including intact immune response these limitations could be overcome in further experiments. Nevertheless, this alternative is also challenging with long time requirement for tumour development²⁵ as well as inability to study metastases due to heavy multifocal primary tumour load which urges premature killing of the

Figure 2. Correlation of BLI signal and CBCT tumour volume for A549 and H1703. Data points (dots) were fitted by linear regression analyses for (2a) A549 ($n = 4$) and (2b) H1703 tumours ($n = 2$). BLI, bio luminescence imaging; CBCT, cone beam CT.

2a

2b

mice.²⁶ Another argument is the missing opportunity to investigate patient-relevant target-specific treatments with genetically engineered mouse models.

A further goal of our study was to characterise the sensitivity and comparability of two different imaging modalities (BLI and CBCT) to decide whether to use them in combination or separately for optimal longitudinal growth monitoring. Furthermore, we investigated histological features of the individual orthotopically grown tumours as well as their microenvironmental parameters (hypoxia, vessels and necrosis) to understand the growth pattern.

Prior studies used a relatively large number of cells in a large volume of cell suspension, which was associated with early metastatic spread from the lung to thorax or axillary lymph nodes. Therefore, these models often did not represent a localised lung tumour,^{27,28} which is a prerequisite for studying an experimental curative therapy such as radiotherapy. Our spheroid transplantation model (H1703) showed initially localised lesions (Figure 1a), which grew slowly over time with a suitable period

before metastatic spread, enabling the initiation of a curative therapy within this time window. The transplantation technique was easy, not time consuming without procedure-related mortality or complications.

As not all cell lines develop spheroids,¹⁰ we tested another method of cell transplantation in these cases. In agreement with already published data,⁹ we injected a dramatically reduced number of cells (A549) in a small cell suspension volume (50,000 cells in 3–5 μ l) with the help of a stereotactic injection system, which allows an accurate depth and location of the injection.⁹ This technique also led to an initially localised lesion with metastatic spread within the same lung (Figure 1h) 5 weeks after transplantation, mimicking the pathological behaviour of NSCLC from single lesions to metastatic disease. In comparison, we observed a relatively early and extensive spread of tumour cells (Figure 1a and h, CBCT) after surgical transplantation of cell suspension. One possible reason could be the use of Matrigel as an anchorage with cell suspension, as it has been seen in a previous study that local tumour growth is enhanced by co-injected Matrigel.²⁹

There are some technical limits of transplantation approaches in our study. The results suggest a likely dependence of orthotopic tumour growth on the integrity of spheroids used for transplantation. The established H23 model showed an early metastatic spread (Figure 1e), which could be explained by a possible disintegration of spheroids during the transplantation process. On the other hand, we noticed spillage of cell suspension in some cases along the puncture channel during stereotactic injection, which is the reason for the development of subcutaneous nodules at the injection site. Taking all these factors together, we would recommend a transplantation of spheroids with the help of a stereotactic system (20G Hamilton syringe) in order to overcome the mentioned limitations.

A second aim of our study was the systematic comparison of different imaging modalities for real-time monitoring of orthotopic lung models. Non-invasive BLI technology was selected to investigate longitudinal tumour growth, as it has been shown to enable sensitive and qualitative imaging in several previous pre-clinical studies.³⁰ Furthermore, CBCT was performed to identify the exact anatomical localisation of the tumour as well as its volume and shape. In an exploratory study on a limited number of orthotopic lung tumours we identified a better contrast after injection of contrast agent prior to CBCT, especially in the peripheral region of the lesion. Thus, we decided to use contrast-enhanced imaging for accurate delineation. BLI and CBCT were successfully applied to monitor tumour growth. In agreement with a previously published study on pre-clinical glioblastoma models,³⁰ we identified a significant correlation between BLI obtained signal intensity and measured tumour volume with CBCT in the tumour model A549 (Figure 2a), indicating sensitivity and reliability of BLI in this model. In contrast, for H1703 no significant correlation between both imaging techniques was found (Figure 2b), probably due the smaller sample size. Nevertheless, BLI is known to represent primarily qualitative information instead of quantitative data especially in an organ such as lung, which is anatomically deeply seated with

Figure 3. Histological features of the different orthotopic NSCLC models. (3a) Haematoxylin/eosin staining of murine lungs demonstrates presence of tumour lesions (yellow arrows). The black arrows indicate necrotic zones. (3b) Proliferation rate of three different NSCLC models. Ki-67 staining identified proliferating (brown cells) cells in all models and demonstrates their human origin. (3c) Quantification of total numbers of Ki-67 in 10 microscopic high-power fields (40x magnification) per section, error bars: mean $\pm$ SEM, * p < 0.05, **** p < 0.0001. NSCLC, non-small cell lung carcinoma.

different possible depth levels of tumours resulting in different tissue to tissue and tissue to air borders.

The take rates assessing positive imaging and histological data for all used cell lines were promising (H1703–100%, H23–50% and A549–50%). We found a higher sensitivity by determination based on BLI, but lower with subsequent CBCT (see exemplary Figure 1e), which was also in line with a previous study in rats.¹¹ Possible reasons could be early spread of tumour cells at a size that was not captured by CBCT images, development of tumours within atelectasis or subcutaneous metastases at the injection site due to backward travel of tumour cells through the injection channel. Therefore, BLI is most suitable as single imaging technique for detection of orthotopic lung tumours or for growth monitoring as well as treatment planning if a substantial correlation with CBCT volumes is known. However, to define tumour

growth and particularly for generation of spatial anatomical information in uncharacterised models, we recommend to use both modalities together.

Our histological data demonstrated compliance with our imaging data for all models. We could confirm, for example, localised nodular tumours in H1703, extensive early metastatic spread in H23 and late metastatic spread in A549 (Figure 3a). The proliferation index, which was assessed by Ki-67 staining, demonstrated considerable heterogeneity among cell lines (Figure 3b and c), which is also in line with published literature in a clinical setting.³¹ Furthermore, a model-dependent characteristic of microenvironmental parameters could be shown: more hypoxia with less vascularity as well as more areas of necrosis was noticeable in orthotopic A549 tumours and opposite in H1703 models (Figures 3 and 4). Currently, to the best of our knowledge, there

Figure 4. Representative images of microenvironmental staining for H1703 (4a, 4c) and A549 (4b, 4d) tumours. Staining with pimonidazole (green) as hypoxia marker and CD31 (red) for vascularity. Inset in both models showing the magnification of the respective area. Magnification 10x (right) and 20x (left). White and yellow arrows indicate hypoxia and vessels respectively. NC, Negative control.

are no further data available on such a comparison in orthotopic models. However, our findings of significant intertumoural heterogeneity are in line with the already published results of Yaromina et al, in five subcutaneously transplanted squamous cell carcinoma models.¹⁹ On the other hand, though orthotopic models are considered to more realistically mimic the clinical situation with respect to metastases and therapeutic response,^{4,32–34} an orthotopic lung tumour might not be a suitable model to study specific radiobiological issues for example regarding hypoxia. Small orthotopic lesions within normal lung parenchyma may not demonstrate poor oxygenation, which has already been reported by Edward et al.³⁵ These authors have shown that orthotopic A549 tumours are relatively well oxygenated compared to their subcutaneous counterparts.

A systematic comparison of growth patterns and histological/microenvironmental characteristics of subcutaneous and orthotopic tumours on a wide range of models is currently missing. Therefore, our reported results on orthotopic lung tumours could be the basis for such a comparison with subcutaneous counterparts to complete characterisation of these pre-clinical models.

As an outlook, we will also conduct a planning study in these tumours as a next step for the implementation as translational model.

CONCLUSION

We developed orthotopic NSCLC models that resemble biological growth behavior similar to that seen in the clinic. An advantage of using human cancer cell lines is the ability to study the impact of different genomic tumour profiles on local treatment outcomes, in contrast to orthotopic models in genetically engineered mouse models which are more limited with regard to available genotypes.³⁶ Monitoring of orthotopic tumour growth using BLI in combination with high-resolution measurement of tumour volume with contrast-enhanced CBCT and histology analysis offers an attractive model to study the biology of lung cancer longitudinally and to test the efficacy of radiotherapy and new treatment agents. A systematic comparison of microenvironmental parameters with subcutaneous counterparts will contribute to complete characterisation of these pre-clinical models.

ANIMALS

We clarify that our study on animal subjects has been carried out in accordance with current international and national standards and the research procedures were approved by the responsible institutional committee on animal experimentation.

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