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Evaluation of Tumor Micro-Environment in an Animal Model using a Nanoparticle Contrast Agent in Computed Tomography Imaging

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Abstract

RATIONALE AND OBJECTIVES—Non-invasive longitudinal imaging of tumor vasculature could provide new insights into the development of solid tumors, facilitating efficient delivery of therapeutics. In this study, we report three-dimensional imaging and characterization of tumor vascular architecture using a nanoparticle contrast agent and high-resolution computed tomography (CT) imaging.

MATERIALS AND METHODS—Five Balb/c mice implanted with 4T1/Luc syngeneic breast tumors cells were used for the study. The nanoparticle contrast agent was systemically administered and longitudinal CT imaging was performed pre-contrast and at serial time-points post-contrast, for up to 7 days for studying the characteristics of tumor-associated blood vessels. Gene-expression of tumor angiogenic biomarkers was measured using quantitative real-time polymerase chain reaction (qRT-PCR).

RESULTS—Early-phase imaging demonstrated the presence of co-opted and newly developed tumor vessels. The co-opted vessels demonstrated wall-permeability and ‘leakiness’ characteristics evident by an increase in extra-vascular nanoparticle-based signal enhancement visible well beyond the margins of tumor. Diameters of tumor-associated vessels were larger than the contralateral normal vessels. Delayed-phase imaging also demonstrated significant accumulation of nanoparticle contrast agent both within and in areas surrounding the tumor. A heterogeneous pattern of signal enhancement was observed both within and among individual tumors. Gene-expression profiling demonstrated significant variability in several angiogenic biomarkers both within and among individual tumors.

CONCLUSIONS—The nanoparticle contrast agent and high-resolution CT imaging facilitated visualization of co-opted and newly developed tumors vessels as well as imaging of nanoparticle

accumulation within tumors. The use of this agent could provide novel insights into tumor vascular biology and could have implications on the monitoring of tumor status.

Keywords

Computed tomography; imaging; tumor; angiogenesis; blood-pool; nanoparticle; contrast agent

INTRODUCTION

Solid tumors undergo significant changes in their architecture during growth and development. Angiogenesis, the growth of new blood vessels, is one of the hallmarks of growing tumors [1,2]. The tumor blood vessels show distinctly different patterns compared to blood vessels found in normal tissues [3,4]. The presence of large pores in the endothelial lining of the vessels and the absence of continuous pericyte and smooth muscle cell coverage are two of the commonly observed traits of “leaky” tumor blood vessels [5–7]. These abnormalities in tumor vessels often lead to significant changes in the transport processes within the tumor interstitial space, affecting the delivery of nutrients and therapeutics [8]. Rapidly growing tumors also represent a large source of growth factors such as vascular endothelial growth factor (VEGF) and its receptors, e.g. VEGFR-1 and VEGFR-2, that are then systemically distributed [9]. The increased levels of growth factors and their receptors are known to have implications for blood vessel permeability, rapid proliferation, and the growth and metastatic potential of tumors. Furthermore, while tumors of different origin are expected (and known) to demonstrate marked variability in tumor-architecture and biomarker levels, inter- and intra-tumor heterogeneity has been observed even within the same tumor type [10–12]. This heterogeneity could have significant implications in personalized tumor treatment.

Non-invasive monitoring of tumor architecture coupled with gene-expression profiling could therefore enhance our understanding of growing tumors and its interactions with the surrounding host tissue. Dynamic and longitudinal imaging could also provide insights into the functional characteristics of these tumor blood vessels. One methodology that has been used in pre-clinical models of solid tumors is non-invasive micro-CT imaging [13–15]. The 3D nature of the technique combined with high spatial resolution provides excellent assessment of tumors. The linear relationship between image signal enhancement and contrast agent concentration enables accurate quantitative assessment of tumor vasculature and its micro-environment. However the pre-clinical utility of CT imaging for investigating tumor vasculature has been limited due to two major limitations of conventional contrast agents: their rapid systemic clearance from the blood pool and low vessel conspicuity due to high propensity to rapidly extravasate into the tumor extra-vascular space. In this work, we evaluated the tumor micro-environment using a nanoparticle contrast agent and CT imaging. Specifically, we investigated whether a long circulating blood-pool nanoparticle CT contrast agent can enable (1) visualization of tumor vasculature and (2) demonstration of tumor vessel “leakiness” by imaging nanoparticle extravasation into the tumor interstitial space. Early phase imaging was performed to investigate the feasibility of tumor vascular imaging. Longitudinal delayed-phase imaging was performed to study tumor vessel permeability as well as the transport and accumulation of nanoparticles in tumors. These studies were performed during the NCI-sponsored Cancer Imaging Summer Camp, held at Washington University, St. Louis, in June 2009.

MATERIALS AND METHODS

Fabrication of nanoparticle contrast agent

A concentrated solution of iodixanol (550 mg/ml) was prepared by dissolving iodixanol powder in distilled water at 60 °C. The nanoparticle contrast agent, also referred to as liposomal-iodine, was prepared using a procedure described earlier [16,17]. Briefly, a lipid mixture (150 mM) consisting of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) (Lipoid, Germany), cholesterol (Lipoid, Germany), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (DSPE-mPEG-2000) (Lipoid, Germany) in a 55:40:5 molar ratio was dissolved in ethanol. The ethanol solution was hydrated with the iodixanol solution and then sequentially extruded on a Lipex thermoline extruder (Northern Lipids Inc., Canada) to size the liposomes to ~ 100 nm. The resulting solution was diafiltered using a MicroKros^R module (Spectrum Laboratories, CA) of 500 kDa molecular weight cut-off to remove un-encapsulated iodixanol. The size distribution of liposomes in the final formulation was determined by dynamic light scattering (DLS) using a ZetaPlus Analyzer (Brookhaven Instruments, UK) at 25 °C. The iodine concentration in the final liposomal solution was quantified by spectrophotometry (λ_{Abs} at 245 nm).

In vivo studies

Animal tumor model—Animal studies were performed under a protocol approved by the institutional animal welfare committee. Four to six weeks old Balb/C mice (average weight 18 gm, weight range: 16–20 gm) were used for the study. Syngeneic breast tumors were grown in mice by orthotopic implantation of 500,000 4T1/Luc cells in the right flank of the mice at about 10–12 days before start of imaging studies. The tumors were 8–10 mm in diameter at the start of imaging studies.

Micro-CT setup—Imaging was performed using an Imtek microCAT-II micro-CT system (Imtek Inc., TN). Imaging parameters were 60 kVp, 500 μ A, 250 ms/exposure, 400 views acquired over a 360° rotation. A typical scan took approximately 15 minutes to complete. The radiation dose associated with each scan was 3.6 cGy. Volumes were reconstructed in a matrix of 1280x512x512 at 80 μ m isotropic voxel size.

Imaging study—A total of five mice were used for the study. For the imaging studies, the nanoparticle contrast agent was slowly infused via the tail vein at a dose of 23 μ L/gm of body weight. During imaging, the animals were maintained under anesthesia with 2–3% isoflurane. Imaging was performed at various time points as described in Table 1. Longitudinal imaging was performed in all animals at serial time points with the last image acquisition performed on day 7 post-administration of the nanoparticle contrast agent.

Quantitative RT-PCR

Gene-expression analysis was performed using quantitative real-time polymerase chain reaction (qRT-PCR) to study inter- and intra-tumor heterogeneity of angiogenic biomarkers. Animals were euthanized with an overdose of isoflurane after the final imaging session, and the tumors extracted. Care was taken to maintain the orientation of tumors by placing anatomical markers representative of posterior and ventral regions. Tumor tissue was perfused with nuclease-free phosphate buffered saline (PBS) and snap frozen in liquid nitrogen. Tumors were sectioned into four quadrants and total RNA was extracted from tissue sections weighing ~155mg (from tumors 1,2 and 3) and ~40mg (from tumor 4) using RNeasy Midi and Mini-RNA extraction kits (Qiagen, CA) according to the manufacturer's protocol. Total RNA extracted from flank associated muscle tissue served as the reference sample to comparatively determine fold changes in gene expression in the respective tumor

tissues. Primer Express 3.0 software (Applied Biosystems, CA) was used to design primer sets to amplify *Mus musculus* angiopoietin-1, angiopoietin-2, vascular endothelial growth factor A (VEGF-A), Fibromyalgia Syndrome (FMS)-like tyrosine kinase-1 (Flt-1) and kinase insert domain protein receptor (Flk-1) targets; and hypoxanthine guanine phosphoribosyl transferase 1 (Hprt1) endogenous control using mRNA sequences obtained from GenBank. Extracted total RNA was quantified, reverse transcribed into cDNA and ~20ng cDNA equivalents of total RNA were used in qRT-PCR reactions according to methods previously published [18]. Briefly, primer sets were validated for PCR efficiencies using six cDNA concentrations (1, 2, 5, 10, 20 and 50 ng). qRT-PCR was performed using an Step-One-Plus real-time PCR system (Applied Biosystems, CA) in 20µl reactions consisting of cDNA, Power^R SYBR Green PCR Master Mix (Applied Biosystems, CA) and forward and reverse primers at 0.9 µM concentrations. Samples were analyzed in triplicate and data expressed as relative fold changes when compared to the reference sample. Four tumors (T1, T2, T3, T4) were used for qRT-PCR analysis. One tumor (T5) was processed for immunohistology.

Immuno-histology

After the final imaging session, T5 was extracted and perfused with PBS. The tumor was stored frozen until ready for processing. Hematoxylin and eosin (H&E) staining was performed on four micron thick frozen sections to survey tumor morphology. Adjacent sections were stained with endothelial marker fluorescein isothiocyanate (FITC)-conjugated CD-31 to observe tumor vessels. Macrophage staining of tumor tissue sections was performed using FITC-conjugated Mac-2.

Image analysis

All data was transferred to an Apple MacPro workstation for image analysis. Thick-slab maximum intensity projection (MIP) images in axial, coronal and sagittal plane were created in ImageJ (v-1.41o). 3D volume-rendered images were created in Osirix (v-3.6, 64-bit) for visual representation.

Data analysis

Data analysis of all image sets was performed in ImageJ (v-1.41o). For pharmacokinetic analysis, contrast enhancement was measured at regions of interest (ROI) in blood-filled chamber of the left ventricle, liver, spleen, kidney, bladder and muscle. ROIs were drawn in 2D axial images at three different locations for each measurement. For the organs, care was taken to not include vascular area within the ROIs. Results were presented as time-attenuation plots with average values and standard deviations reported in Hounsfield units (HU). The blood clearance rate constant of the nanoparticle contrast agent was determined using a one-compartment model. The visualization of tumor vasculature was assessed by quantitative analysis of pre-contrast and post-contrast images (T=0hr and T=24hr). Additional post-contrast time points were not included due to difficulty in separating vessel signal from perivascular extravasated signal. For the analysis, region of interests (ROIs) were drawn on 2D axial slices. Due to small intra-tumoral vessels, square ROIs were drawn encompassing a vessel. Maximum signal intensity within each ROI was used for the analysis. Three ROIs were considered for each vessel and three different vessels were considered within each tumor. Results were presented as average values and standard deviation reported in Hounsfield units (HU). For quantitative analysis of nanoparticle extravasation, tumor ROIs were drawn on 2D axial slices. Seven ROIs were drawn on different slices to span the entire tumor. Results were presented as average values and standard deviation reported in Hounsfield units.

Measurements of vessel diameter were performed using both 2D axial images as well as thick-slab maximum intensity projection (MIP) images. In some cases, the MIP images enabled visualizing the continuity of tumors vessels, thus facilitating the drawing of cross-section lengths for measuring vessel diameter. Vessel cross-section length measurements were performed for both tumor-associated vessels and contra-lateral normal vessels. Measurements were performed at three locations per vessels and the values were reported as average vessel diameters with standard deviations.

Statistical analysis

A two-tailed t-test was performed for statistical analysis of tumor vasculature visualization and nanoparticle extravasation. One-way ANOVA with post-hoc Bonferroni test was performed to determine the significance of the relative gene expression levels within different quadrants of the tumors. For both of the above analysis, a p-value of less than 0.05 was considered statistically significant.

RESULTS

The nanoparticle contrast agent demonstrated long blood circulation times with an *in vivo* half-life of 41 hr and a blood clearance rate constant of $0.0179 \pm 0.0014 \text{ h}^{-1}$. A strong vascular signal ($983 \pm 23 \text{ HU}$) resulting in high tumor micro-vessel conspicuity was achieved in the early phase of the imaging (Figure 1a). The noise level in the images, measured as standard deviation in a region of interest in air, was $70 \pm 4 \text{ HU}$. The vascular signal returned to baseline about 120 hr post-administration, indicating clearance of the nanoparticle contrast agent from the blood pool. Liposomal nanoparticles such as those used in this agent are known to be cleared via the reticulo-endothelial system (RES). Thus, the contrast progressively enhanced in the liver and spleen with the liver signal steadily increasing upto 120 hr before beginning to decline (Figure 1a). This correlated with the transient behavior in blood where the signal returned to baseline at about 120 hours. The transient profile of signal enhancement in the spleen also demonstrated similar behavior. The absence of signal in kidneys and bladder indicated *in vivo* stability of the nanoparticle contrast agent (Figure 1b).

Early phase imaging demonstrated excellent visualization of tumor micro-vasculature in all the animals (Figure 2). The nanoparticle contrast agent enabled clear depiction of tumor vessels upto 24 hr post-contrast ($p < 0.05$) (Figure 3a). At delayed time-points, vessel identification was confounded due to significant peri-vascular signal arising from the extravasated nanoparticles. Most of the tumors demonstrated growth of co-opted vessels. The origin of co-opted vessels was traced to major venous structures including the inferior vena cava or the jugular vein. Interestingly, arterial co-option of vasculature could be not detected in any of the tumors. A majority of the co-opted vessels also demonstrated high permeability and 'leakiness' characteristics as observed in the delayed-phase imaging (Figure 4). This resulted in nanoparticle extravasation into the peri-vascular space as confirmed by continuous increase in peri-vascular signal beyond the boundaries of tumor margins detected as early as 48 hours after administration of the nanoparticle contrast agent. In addition to co-opted vessels, several newly developed vessels were also visible in the tumors (Figure 5). All vascular structures associated with the tumors i.e., co-opted, newly developed and existing vessels, demonstrated 30–60 % significantly larger vessel diameters and higher tortuosity compared to the contra-lateral normal vessels (Figure 5) (Table 2).

Delayed phase imaging also enabled probing of the intra-tumoral vasculature for leakiness characteristics to nanoparticles (Figure 6). In the tumors, extravasation and accumulation of the nanoparticle contrast agent was seen as early as 24 hr post-administration and continued to increase over time. Statistical significance in extravasation of nanoparticle contrast agent

within the tumor tissue was demonstrated in all the animals ($p < 0.05$) (Figure 3b). There was significant variability in signal enhancement, both among different tumors as well as within individual tumor (Figure 7). A peripheral rim of highly vascularized enhancement was observed in most of the tumors. The enhancement pattern continued to increase over time, indicating extravasation of nanoparticle contrast agent into the peri-vascular area and followed by convection-based transport deeper into the interstitial space. Areas of diffuse and distinct signal enhancement were observed within all the tumors. In addition to the outer peripheral area of enhancement, a second ring was observed in some of the tumors. Most of tumor cores demonstrated little or no enhancement.

Immuno-histology data correlated well with CT data (Figure 8). An extensive network of blood vessels was observed in the periphery of the tumor as evident from CD-31 staining of endothelial cells. Hematoxylin and eosin (H&E) staining demonstrated the presence of a necrotic core in the center of the tumor. A small fraction of degenerating and apoptotic cells was also observed throughout the tumor mass. Very few macrophages were observed in the tumor interstitial space as confirmed by Mac-2 staining. Macrophages were however observed infiltrating the vessel lumen in some of the tumor vessels.

Quantitative RT-PCR indicated elevated levels of VEGF-A, VEGFR-1 and VEGFR-2 in most of the tumors (Figure 9). Among the Tie-receptor ligands (Angiopoietin-1 (Ang-1) and Angiopoietin-2 (Ang-2)), elevated levels of Ang-2 were observed in all the tumors. Significant heterogeneity in gene-expression levels was observed among tumors for all the biomarker genes except Ang-1 (p -value < 0.05). Significant heterogeneity in gene-expression levels was also observed within each tumor except for Ang-1 in T-1 and VEGF-A and VEGFR-2 in T-4 (p -value < 0.05).

DISCUSSION

Tumor vasculature is one of the most important targets in the treatment of solid tumors. Over the past decade, several new cancer therapeutics have been tested in the pre-clinical and clinical domain for targeting tumor vasculature [19–21]. The development of tumor vasculature occurs through a highly complex set of interactions between several cellular and molecular players [22,23]. A unified understanding of the heterogeneous tumor vascular network, its micro-environment and functional properties would provide new insights about tumor vascular biology that could enable the development of efficient drug delivery strategies.

Non-invasive high resolution CT imaging can enable longitudinal interrogation of tumors thus providing three-dimensional dynamic and functional information [24–26]. The high-spatial resolution combined with the use of contrast agents can provide insights into the vascular network of developing solid tumors. In addition, the linear relationship between iodine concentration and signal enhancement in the image could enable accurate quantitative assessment of tumor vascular parameters such as vascular permeability rate and vascular volume fraction [27]. Despite the benefits of CT imaging, its use has been limited in the pre-clinical setting. Some of the major limitation arises from the physico-chemical properties of conventional iodinated contrast agents. First, the short *in vivo* half-life of conventional contrast agents results in their rapid clearance from systemic circulation. In the clinical setting, this problem is overcome by bolus systemic injection of contrast agent combined with rapid imaging. However, this approach is not feasible in the pre-clinical domain due to the inherent long scan times (10 – 20 mins) associated with a majority of small animal imaging CT scanners in order to achieve high spatial resolution [16,28]. Secondly, due to their sub-5 nm size, iodinated contrast agents have a high propensity to extravasate not only through permeable tumor blood vessels but also through normal capillaries. Consequently,

vessel conspicuity is significantly degraded in scans obtained with conventional contrast agents due to low contrast-to-noise ratio. These limitations can be overcome using novel contrast agents that remain primarily intravascular and have long blood residence times [16]. In this work, we probed the tumor vascular architecture and its micro-environment using CT imaging and a long circulating nanoparticle blood pool contrast agent. High-resolution spatial imaging combined with uniform and stable signal enhancement enabled excellent three-dimensional visualization of the tumor vasculature during early-phase imaging. The long *in vivo* blood residence time of the nanoparticle contrast agent enabled longitudinal tracking, thus allowing visualization of regions with high 'leakiness' and increased vessel permeability as demonstrated by extravascular signal enhancement. The tumors demonstrated extravascular signal enhancement as early as 24 hours post-administration of the nanoparticle contrast agent. Furthermore, highly heterogeneous profiles of signal enhancement were observed in delayed-imaging sessions within all tumors. The diffuse pattern of signal enhancement suggests extravasation of nanoparticle contrast agent into the peri-vascular region of the interstitial space followed by deeper transport into the interstitial space. The tumor enhancement profiles also varied from animal to animal, demonstrating both inter- and intra-tumor variability. Most interestingly, the presence of co-opted and newly developed blood vessels associated with the tumor was demonstrated. The co-opted blood vessels also demonstrated highly permeable and 'leakiness' characteristics, suggesting that neo-angiogenesis takes place beyond the tumor margins itself.

The tumor blood vessels were also tortuous and dilated compared to normal vessels. The vessel diameters of venous structures within the tumor were as much as 90 % larger compared to contra-lateral normal vessels. Previous studies have demonstrated high tortuosity in tumor vasculature compared to normal blood vessels [29]. Furthermore, the relatively higher over-expression of Ang-2 compared to Ang-1 could suggest a stronger effect of Ang-2 in destabilizing the vascular architecture since previous studies have demonstrated that over-expression of Ang-1 prevents tumor vessel dilation [30]. The small size of tumor arteries precluded our analysis of studying their vessel diameters. Most of the tumors also demonstrated vessel co-option. It is interesting to note that there was no evidence for arterial vessel co-option, while there were clear examples of venous co-option. This raises the question of the origin of tumor blood supply. Thus it appears that neo-angiogenesis must be occurring on the upstream or arterial side of the tumor. The inability to visualize arterial side vessel co-option could be attributed to their smaller diameters (probably < 50 μm) which would require both higher spatial and contrast resolution, achievable in principle with micro-CT but involving higher x-ray radiation dose.

In a recent study using the long circulating nanoparticle contrast agent in a rat breast tumor model, it was demonstrated that the degree of tumor vascular permeability to the nanoparticle contrast agent correlates with gene-expression levels of VEGF and its receptors [18]. While tumor signal heterogeneity was observed in that study, it could not be investigated on a spatial-basis due to the inherent two-dimensional nature of X-ray mammography. In this study, CT imaging enabled acquisition of three-dimensional image data. Quantitative RT-PCR analysis was therefore performed on a spatial-basis, albeit at macroscopic level, to study tumor heterogeneity in both signal enhancement and gene-expression levels of angiogenic biomarkers. Two sets of angiogenic factors were studied: VEGF-A and its receptors (VEGFR-1 and VEGFR- 2) and angiopoietins (Ang-1 and Ang-2). The role of VEGF-A and its receptors has been extensively studied in the context of tumor blood vessel growth and vascular normalization [31]. Angiopoietins are ligands for the Tie2 receptor, a member of the endothelial cell type receptor tyrosine kinase family implicated in endothelial cell survival and vessel maturation [32–34]. Ang-1 plays the role of an agonist ligand mediating stabilization and maturation of developing vessels. On the other hand, Ang-2 is an antagonist ligand known to de-stabilize the function of Ang-1, thus

de-stabilizing tumor vasculature [35]. Several studies have demonstrated the collaborative and complementary roles of VEGF and angiopoietins during tumor angiogenesis [36–38]. Interestingly in our study, the expression levels of various angiogenic biomarkers were not only elevated, but also varied significantly within each tumor. Among the VEGF group, elevated levels of VEGF-A was observed in the majority of tumors. Expression levels of VEGFR-1 and VEGFR-2 also varied significantly within and among different tumors. Among the angiopoietins, high levels of Ang-2 were observed in each of the quadrants of all tumors. Elevated expression levels of VEGF-A and Ang-2 and simultaneous reduced expression levels of Ang-1 have been known to cause rapid growth of tumor blood vessels [39]. The high expression levels of Ang-2 together with venous vessel co-option and the highly permeable nature of the coopted vessel could have important implications. Previous studies have demonstrated that elevated Ang-2 levels in the venous system can be a useful predictor of tumor invasiveness and prognosis in human hepatocellular carcinoma [40]. The leaky venous vessels could therefore be potential routes for migration of tumor cells to distant sites of metastasis. The ability to non-invasively monitor such changes in tumor vascular biology could provide new insights in understanding tumor growth and metastasis.

In addition to studying the tumor vascular biology, the utility of the nanoparticle contrast agent could be expanded to monitoring tumor status. In a previous study, the use of nanoparticle contrast agent as a ‘physiomarker’ for investigating the angiogenic potential of tumors had been demonstrated in a breast cancer model [18]. This could have implications both in patient-specific chemotherapy and in assessment during therapy monitoring. Furthermore, the utility of nanoparticle contrast agent as a prognostication tool to assess nanoparticle-based chemotherapy of breast tumors using 2D mammography imaging has also been demonstrated [17]. The availability of such imaging tools could enable patient selection from a general population that would respond to nanoparticle-based chemotherapy and therefore significantly reduce the percentage of non-responders who would otherwise undergo the rigors of such a treatment.

Several limitations exist in the current study. The use of quadrant-based PCR analysis limited the number of genes that could be correlated with the CT-derived signal enhancement. Future studies will therefore consider incorporating mass-spectrometric proteomic analysis or micro-array based proteomic analysis to identify gene expression over a broad spectrum, and its correlation to signal enhancement. Although the VEGF family and angiopoietins (Ang-1 and Ang-2) have been extensively studied in the context of tumor biology, several other genes could play a critical role in tumor permeability which could result in the observed nanoparticle contrast agent leakage. Thus, future studies should also consider investigating other angiogenic factors that could play a critical role in the observed vessel leakiness. The small cohort of animals used in the study limits the generalization of the results to other types of developing tumors. An understanding of the evolution of vessel co-option as well as its morphology and characteristics in response to tumor growth is clearly warranted.

However, the current study provides insights into the functional and dynamic role of tumor vascular architecture. Monitoring the leakiness of co-opted and neo-angiogenic vessels to anti-angiogenic therapeutics could provide new approaches in the treatment of solid tumors. Furthermore, the combination of CT imaging and the nanoparticle contrast agent provides a new set of tools to study not only tumor biology but also how nanoparticles interact with the tumor micro-environment. This would augment our understanding of nanoparticle-based chemotherapy and therefore facilitate the development of more effective and efficient strategies for delivering anti-cancer agents to solid tumors, thus enabling personalized cancer therapy.

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Figure 1. *In vivo* pharmacokinetic profile and bio-distribution of nanoparticle contrast agent

The nanoparticle contrast agent demonstrated long blood residence times. The signal returned to baseline at about 120 h (5 days) post-administration. The agent is primarily cleared by the liver and spleen as indicated by steady signal increase in these organs (A). Negligible signal enhancement is observed in the kidney, bladder and muscle indicating *in vivo* stability of the nanoparticle contrast agent (B).

Figure 2. Early-phase imaging of co-opted and neo-angiogenic tumor vessels

Top-row: coronal 3D volume rendered images demonstrating co-opted vessels (white arrows) and newly developed tumor vessels (green) in early phase imaging (immediately after nanoparticle contrast agent administration). Both the co-opted and newly developed vessels traced their origin back to major venous structures such as the jugular vein (origin of vessel labeled with white arrow) or the inferior vena cava (origin of vessel labeled with green arrow). Corresponding blood vessels on the contra-lateral side (blue arrow) were either absent or not clearly demonstrated. *Bottom row:* coronal maximum intensity projection (MIP) images showing an expanded view of the tumor and surrounding regions demonstrating the tumor vasculature.

Figure 3.

(A) Analysis of tumor vessel attenuation in pre-contrast and post-contrast images. The nanoparticle contrast agent enabled visualization of tumor vasculature as demonstrated by statistical differences in pre-contrast and post-contrast measurements ($p < 0.05$). (B) Longitudinal analysis of tumor signal enhancement. The nanoparticle contrast agent enabled visualization of tumor vessel leakiness as demonstrated by significant signal enhancement in tumor at delayed time-points ($p < 0.05$).

Figure 4. Dynamic characteristics of co-opted tumor vessels

Coronal volume-rendered images demonstrating the architecture of co-opted tumor vessels (images A, B) (yellow arrow). The contra-lateral vessel demonstrated limited development (blue arrow). The path of the contra-lateral vessel could not be traced below the thoracic region on 2-D images. Dynamic monitoring of nanoparticle contrast agent extravasation from the co-opted vessel (images C – G) demonstrated significant extravasation of nanoparticle contrast agent from the co-opted vessel, well beyond the margins of tumor, thus implicating the highly permeable and ‘leaky’ nature of the co-opted vessel. The tumor also demonstrate heterogeneous pattern of signal enhancement suggesting highly permeable intra-tumoral vessels.

Figure 5. Development of tumor venous system

Coronal maximum intensity projection (MIP) image comparing the structures of tumor-associated vessels (TV) versus contra-lateral normal vessels (NV). The tumor venous structures were highly dilated and tortuous compared to their counterparts on the contra-lateral side. Micro-CT imaging was performed immediately after the injection of the nanoparticle contrast agent.

Figure 6. Dynamics of tumor signal enhancement

Coronal 3D volume-rendered images demonstrating the extravasation and accumulation of nanoparticle contrast agent within the tumor (yellow arrow). Immediately after administering the nanoparticle contrast agent, the overall body and tumor vasculature is nicely demonstrated. Tumor accumulation of nanoparticle contrast agent was observed as early as 24h. No image-detectable nanoparticle contrast agent signal was observed from the blood pool at post-120h (day-5) as confirmed by the absence of any vessels or heart signal on post-120h image. However, the tumor is clearly enhanced. The only other organs enhanced are the liver and spleen which are the organs for nanoparticle contrast agent clearance.

Figure 7. Signal enhancement pattern in tumors

Coronal thick-slab (2 mm) maximum intensity projection (MIP) images demonstrating signal enhancement patterns at different locations in two tumors. Imaging was performed on day-5 (1 A-H) and day-7 (2 A-H) post nanoparticle contrast agent administration.

Heterogeneous signal enhancement pattern was observed between the tumors as well as within each tumor. Peripheral enhancement indicating nanoparticle contrast agent extravasation from highly permeable vessels was observed. A second inner circular ring of enhancement was observed within the tumor surrounding non-enhanced central core (1-C, 1-D). A region of high nanoparticle contrast agent accumulation, corresponding to large signal enhancement was also observed near the surface (blue arrow).

Figure 8. Comparison of CT image enhancement with immuno-histology

(A) CT image demonstrating negligible enhancement in the tumor central core and significant enhancement surrounding it; (B) Representative hematoxylin and eosin (H&E) staining demonstrating the presence of a central necrotic center and viable region surrounding it; (C) CD-31 staining demonstrates the presence of extensive vessel growth mainly on the periphery of tumor (20x magnification). (D and F) Mac-2 staining demonstrates infiltration of macrophages in the vessel lumen. Negligible macrophages were seen in the tumor interstitial. (E) Representative H&E staining for images D and F.

Figure 9. Gene-expression profiling of tumors using quantitative real-time polymerase chain reaction (qRT-PCR)

Each tumor was divided into four quadrants based on anatomical markers that were added immediately after the final session of CT imaging. The gene expression levels of various angiogenic biomarkers were then determined for each quadrant using qRT-PCR. Expression levels were normalized to muscle. Error bars represent standard errors.

Table 1

Imaging time points and experimental parameters used in the study.

Animal No.	Animal weight (g)	Iodine Dose (mg I/g)	Imaging Time Points								
			Pre	0h	1d	2d	3d	4d	5d	6d	7d
T-1	16	2.9	X	X		X	X	X			X
T-2	19	3.2		X		X	X			X	
T-3	20	3.2	X	X	X	X			X		
T-4	18	2.9	X	X	X			X			
T-5	19	3.0	X	X	X			X			

Table 2

Comparison of vessel diameters between tumor-associated vessels and contra-lateral normal vessels. Two types of vessel were analyzed based on their origin. For tumors vessels that originated from *inferior vena cava* (IVC), size analysis was performed for vessel segments surrounding the tumor tissue. Identical contra-lateral normal vessels were used for comparison. For co-opted vessel originating from the jugular vein, size analysis was performed for vessel segments in the thoraco-abdominal region. Identical contra-lateral normal vessels were used for comparison. In most animals, the contra-lateral vessels originating from the jugular vein was not clearly demonstrated below the abdominal region. Values are reported as mean and standard deviation with three to five measurements per vessel.

Tumor I.D.	Vessel Origin – Inferior Vena Cava		Vessel Origin – Jugular Vein	
	Tumor vessel diameter (µm)	Contra-lateral vessel diameter (µm)	Co-opted vessel diameter (µm)	Contra-lateral vessel diameter (µm)
T-1	617 ± 66	429 ± 67	448 ± 49	338 ± 9
T-2	696 ± 28	472 ± 3	442 ± 30	381 ± 17
T-3	654 ± 61	473 ± 117	445 ± 32	231 ± 37
T-4	805 ± 42	467 ± 33	194 ± 35	Not visible
T-5	642 ± 82	445 ± 27	336 ± 72	231 ± 41