Lymph Node Metastasis Depends Upon Lymphatic-Convective Transport, Lymphatic Vessel Density and Invasive Phenotype

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INTRODUCTION: The emerging role of tumor-associated lymphangiogenesis in promoting regional lymph node metastasis [1] has resulted in a critical need for dynamic *in vivo* functional assays of lymphatic function in tumors. There is however, a paucity of assays capable of linking the expression of lymphangiogenic cytokines to their corresponding downstream functions. Several approaches have been used to probe the interstitial-lymphatic continuum i.e. transport within the extravascular space in tumors which includes the extracellular matrix (ECM) and lymphatics [2]. These have been responsible for recent insights into lymphatic physiology within tumors. Among the unresolved questions regarding "functionality" of tumoral lymphatics is whether cancer cells in a primary tumor are transported to remote sites via patent, intra-tumoral lymphatic channels. Recently, we developed a noninvasive technique for identifying potential lymphatic-convective channels in a human breast cancer model *in vivo*, using MRI [2]. Here, for the first time we characterize lymphatic-convective transport as well as angiogenic parameters simultaneously *in vivo*, using contrast-enhanced MRI in conjunction with fluorescent microscopy. We found large differences in these parameters between invasive and non-invasive breast tumor xenografts. The functional MRI and optical imaging data together with lymph node and lung metastasis data indicate that although lymphangiogenesis and angiogenesis may be necessary, concomitant possession of the "invasive" phenotype enhances the probability of metastases.

METHODS: Five MCF-7 and five MDA-MB-231 tumor-bearing (>200mm3) mice were scanned using MRI. Multi-slice T1 relaxation rates of the tumor were obtained by saturation recovery combined with SNAPSHOT FLASH. Eight (1mm) slices were acquired with a 256×256μm^2 resolution for three relaxation delays (100, 500 and 1000ms). Images were acquired in two "phases" corresponding to the biphasic kinetics of the MMCA. The "early phase" comprised of images obtained before i.v. administration of 0.2ml of 60mg/ml albumin Gd-DTPA (or biotinylated-albumin-GdDTPA) in saline and repeated every 7 min, starting at 3 min post-injection, up to 31 min. Since drainage of the MMCA either by convection or by lymphatics, is a slow event [2], a second block of MR data was acquired up to 140 min post contrast. After each study, mice were sacrificed and blood T1's determined from tail vein samples. Parameters describing vascular and extravascular transport of MMCA in MCF-7 tumors were calculated from biphasic MMCA tissue concentration-time curves using a novel multiple regression approach [3] that allowed us to compute: vascular volume (VV), permeability-surface area product (PS), fluid transport volume, flux rates, as well as enabled classification of voxels as being either *pooling* or *draining*. Tumor sections were stained for lymphatic vessels (LYVE-1), blood vessels (CD34) and MMCA (biotinylated-albumin-GdDTPA). For each animal, representative slides were scored on a scale ranging from 1 (i.e. few LYVE-1 +ve structures) to 5 (i.e. large number of LYVE-1 +ve structures). Scoring was conducted for 20× fields, and ROIs further classified as "intratumoral" and "peripheral". The Mann-Whitney U test was employed for all statistical comparisons.

RESULTS: **Fig. 1** illustrates differences in MMCA drain between the two tumor types. Overall, there was a greater percentage of draining voxels (9.4±5.8) identified for MDA-MB-231 tumors compared to MCF-7s (2.9±1.5) while the number of pooling voxels was comparable**.** For the two tumor types, although there was no significant difference in VV (6.1±1.7 vs. 4.6±2.1μl/g), the PS for MDA-MB-231 tumors (0.7±0.2μl/g.min) was significantly (p=0.02) higher than for the MCF-7 tumors (0.4±0.2μl/g.min). The efflux rate (0.35±0.44μl/g.min) was also significantly (p=0.007) greater for MDA-MB-231 tumors than for MCF-7 (-0.33±0.19μl/g.min) tumors, which in turn resulted in significantly (p=0.0013) larger volumes of fluid transport in the former (14.7±1.9μl/g) compared to the latter (-9.25±5.5μl/g) (**Fig. 2**). Significantly (p=0.009) greater numbers (assessed by scoring tissue sections, **Fig 3a**) of dilated, sinusoidal peripheral lymphatics (**Fig. 3b**) were observed in MDA-MB-231 tumors compared to collapsed, tenuous lymphatics found in MCF-7 tumors (**Fig. 3c**). Finally, there was a significantly (Fisher's exact test p=0.02) greater number of cancer +ve proximal axilary lymph nodes (11/12 vs. 0/7) (**Fig. 4**) and a significantly (Fisher's exact test p=0.0001) greater number of cancer +ve lungs (7/13 vs. 1/11) in MDA-MB-231 bearing animals, compared to MCF-7 bearing animals.

DISCUSSION/CONCLUSIONS: The differences *in vivo* vascular parameters measured here are consistent with earlier data for these two tumor models as are the differences in lung metastasis [4]. The larger percentage of draining voxels, higher efflux rates and higher exudate volume for the MDA-MB-231 bearing animals compared to the MCF-7 bearing animals are consistent with elevated VEGF levels of MDA-MB-231 tumor [4] and the resulting hyperpermeability. In addition, for the first time we have demonstrated significant differences in lymphatic-convective transport parameters (using MRI) as well as significant differences in lymphatic vessel density (using fluorescent microscopy) between the two tumor models. This difference in lymphangiogenesis and altered extravascular transport combined with the invasive phenotype of MDA-MB-231 cells [4] was most likely responsible for the significantly higher number of lymph node metastases detected in the MDA-MB-231 bearing mice compared to MCF7 bearing mice. In addition to metastatic deposits in the proximal lymph nodes, several MDA-MB-231 bearing animals exhibited lymph node metastasis in the contralateral axillary lymph node, demonstrating the ability of MDA-MB-231 cells to colonize distant lymph nodes as well. Functional MRI assays of vascular and extravascular events in tumors help bridge the gap between histology and the *in vivo* remodeling of the ECM that typically accompanies tumor progression, providing new insights into the mechanistic aspects of invasion and metastasis.

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