Hypoxia and elevated total choline are associated with 'stem-like' cancer cells in breast cancer xenograft in vivo: an MR, SPECT/CT, and optical study

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Introduction: The discovery of stem-like cell populations in several cancers, that are the most likely to be resistant to therapy and to lead to recurrence and metastasis, has generated tremendous excitement for understanding and treating tumor recurrence and metastasis. Stem-like breast cancer cells are usually identified by (i) $CD44^+/CD24^{-\text{ or low}}$ phenotype, (ii) exclusion of Hoechst 33342 (ABCG2 /BCRP transporter) and rhodamine 123 (MDR1) and, (iii) tumor growth from low cell inoculums (1). Recent studies suggest that hypoxia provides a suitable niche for stem cells to maintain their precursor status (2). In tumors, hypoxia is also a major cause of radiation and chemo-resistance (3). Our focus in this study was to understand the role of hypoxia, choline metabolism, and the tumor microenvironment in creating or harboring stem-like breast cancer cells.

Methods: Studies were performed with MDA-MB-231 human breast cancer cells stably transfected with red-fluorescent tdTomato protein (RFP) expressed under control of the VEGF hypoxia response element (HRE). These MDA-MB-231 HRE-RFP tumors were grown orthotopically in female severe combined immunodeficient (SCID) mice. MR experiments were performed with a Bruker horizontal bore 9.4T dedicated animal MR scanner using a home-built RF resonator for the tumor. 3D MR imaging was performed with a fast spin echo sequence and 3D MR spectroscopic imaging (MRSI) of choline distribution with spin echo CSI sequence (TE/TR = 80/1000ms), 512 spectral points and 12x12x8 spatial matrix (1 mm resolution) with 4 averages. Fluorescence imaging of the tumor was performed *in vivo* with a Xenogen IVIS 200 system and endpoint fluorescence imaging was performed with a fluorescence microscope using fresh 2 mm tumor slices prepared with a tissue slicer. For SPECT/CT imaging mice were administered intravenously with 0.616 mCi of [^{125}I] labeled anti-CD44 antibody in 0.17 mL of saline. At 48 h post injection, SPECT images were acquired on a Gamma Medica X-SPECT scanner in 64 projections at 45 sec/projection (1mm resolution). Following SPECT imaging, a CT scan using 512 projections was performed to obtain a co-registered 3D anatomical reference. Since CD44 is a transmembrane hyaluronic acid receptor that exists in several isoforms as a result of alternative splicing, we performed q-RT-PCR analyses of these isoforms as well as analysis of a region common to all isoforms (CD44 All), under normoxic conditions and following treatment with the hypoxia mimetic cobalt chloride. Q-RT-PCR analyses of VEGF and the ABCG2/BCRP transporter were also performed.

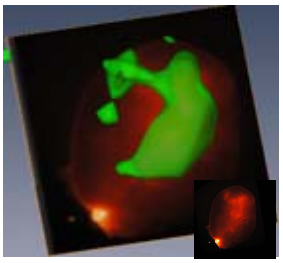

Figure 1a. Overlay of total choline with RFP distribution in an MDA-MB-231 HRE-RFP tumor (~250 mm^3). **Inset** shows RFP expression map only.

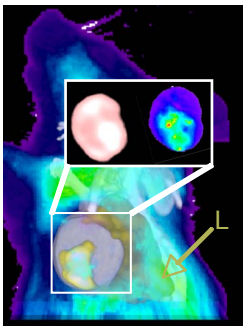

Figure 1b. Images from a representative MDA-MB-231 HRE-RFP tumor showing coregistration of M_0 map of tumor (marked by square) obtained with MRI (gray), RFP expression from Xenogen (blue), and SPECT data (yellow) showing the overlap of a hypoxic region of high fluorescence with high CD44 antibody localization. L: lungs **Inset** shows *ex vivo* SPECT (left) and optical (right) images of a fresh 2 mm thick slice from the tumor.

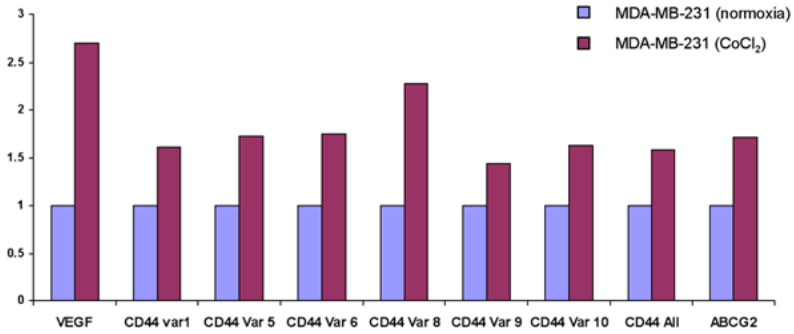

Figure 1c. CD44, VEGF and ABCG2 mRNA expression in MDA-MB-231 cells under normoxic and hypoxic conditions. Hypoxia was achieved by treating cells with 100 micromolar of the hypoxia mimetic cobalt chloride for 24 h.

Results: Consistent with our previous observations that hypoxia regulates choline kinase expression and increases phosphocholine in a human prostate cancer xenograft (4), we observed co-localization of hypoxia (from RFP expression) and total choline in the MDA-MB-231 human breast cancer xenograft model (Figure 1a). These hypoxic regions contained cells with increased expression of the breast cancer stem-cell marker CD44 (Figure 1b) as evident from the higher intensity of ^{125}I radiolabeled anti-CD44 antibody in these regions. Treatment of MDA-MB-231 cells with the hypoxia mimetic cobalt chloride increased expression of CD44 isoforms and total CD44 supporting the possibility that hypoxia may harbor or increase the stem-like phenotype of breast cancer cells (Figure 1c). An increase of VEGF and ABCG2 was also observed (Figure 1c). The increase of VEGF mRNA expression is consistent with the induction of hypoxia in these cells; the increase of ABCG2 mRNA expression is consistent with the increased stem-like phenotype of these breast cancer cells under hypoxic conditions.

Discussion: Our data suggest that hypoxia and elevated total choline may serve as surrogate markers for tumor regions likely to contain stem-like breast cancer cells, and that radiolabeled anti-CD44 antibodies can be used to image stem-like cells *in vivo*. These preliminary data also suggest that stem-like cancer cells may form a dynamic population that may transit between exhibiting stem- and non-stem-like phenotypic characteristics depending upon the tumor microenvironment. Noninvasive imaging becomes particularly important in tracking the dynamics of this population especially for therapies designed to target stem-like cancer cells. Future clinical evaluation of the relationship between elevated total choline using MRSI and $CD44^+/CD24^{-\text{ or low}}$ receptor expression with PET or SPECT in breast cancer patients will further validate these observations.

Acknowledgements: This work was supported by NIH P50 CA103175 and U24 CA92871. We thank Dr. Y. Kato, Ms. Flonne Wildes, Mr. James Fox and Mr Gary Cromwell for valuable technical assistance.

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