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Exploration of melanoma metastases in mice brains using endogenous contrast photoacoustic imaging

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ABSTRACT

Photoacoustic imaging (PAI) provides real time non-invasive and contrast agent free monitoring of some endogenous compounds concentrations that provides improved insights into tissue vascularization and oxygenation which are particularly important during tumor progression.

This study assessed the input of PAI for examination of melanoma brain metastases in an orthotopic mouse model and further focused on spatial analyses within the tumor tissue.

Hemoglobin content appeared to be higher in tumors than in healthy brains. Spatial analyses further showed that angiogenesis was mainly at the tumor periphery. Concomitantly, while healthy brains were highly oxygenated, the tumors were hypoxic and subjected to a gradient of hypoxia from the periphery to the core.

In tumor-bearing brains, spectroscopic PAI clearly revealed the presence of melanin, generating a signal 3 times higher than the background signal in healthy brains. When inserted into tissue mimicking phantoms, the photoacoustic signal of B16F10 melanin-containing cells was linearly correlated to their concentration and the detection limit was 625 cells.

In vivo biological characterization of tumor models by non-invasive imaging of vasculature and tissue hypoxia represents an interesting opportunity for better understanding cancer progression; it is opening new research prospects to improve diagnostic, therapy, and early assessment of tumor treatment efficacy.

1. Introduction

Photoacoustic imaging (PAI) is an emerging technology combining the most compelling features of optical imaging and ultrasound, providing both high optical contrast and high ultrasound resolution at depth in living organisms (Wang et al., 2003a). It consists of illuminating tissues with a pulsed light, in the near-infrared wavelength range where light has its maximum depth of penetration in living tissues. This locally absorbed energy is dissipated into heat, producing a thermoelastic expansion, which generates an ultrasonic wave. This wave propagates through the tissues and can be collected by ultrasound detectors (transducers). Some endogenous compounds such as oxy- and deoxy-hemoglobin, melanin, lipids and collagen, exhibit a specific absorption spectrum together with a photoacoustic effect. By using multiple wavelengths of laser light (spectroscopic PAI) relative concentrations of these specific compounds can be determined (Luke et al., 2013; Wang et al., 2006). This imaging modality thus provides anatomically localized high inherent *in vivo* contrast in several tissue layers, allowing for a precise and non-invasive mapping of several endogenous compounds.

In particular, by detecting the relative amounts of oxy- and deoxy-hemoglobin, oxygen saturation can be calculated ([oxyHb/(oxyHb + deoxyHb)] × 100%) (Wang et al., 2006). It affords improved insights into levels of tissue oxygenation or hypoxia, which is of particular interest in oncology. This principle is applied in many studies postulating that tissues with low levels of oxygenation are more likely to be cancerous according to cancer's known metabolic changes (Warburg effect) and particularly hypoxia in later stages (Cairns et al., 2011; Kim and Dang, 2006).

With primary advantages including clinically relevant imaging depths (up to several centimeters) with submillimeter resolution (Wang and Hu, 2012), real-time monitoring, portability, and lack of ionizing radiations, PAI holds promise as a clinical modality for cancer detection (Valluru et al., 2016). Several applications pertaining to cancer imaging are currently being used in clinical trials for the exploration of microcirculation (via hemoglobin concentration) and tissue oxygen

Abbreviations: PAI, photoacoustic imaging; oxyHb, oxygenated hemoglobin; deoxyHb, deoxygenated hemoglobin; StO_2, tissue oxygen saturation; HbT, total hemoglobin content; EMT, epithelial-mesenchymal transition

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saturation in breast (Heijblom et al., 2016), prostate, and ovarian cancers (Valluru et al., 2016). Another attractive application of PAI is the detection and staging of skin cancer, given the potential advantages of this technology for non-invasive melanin endogenous contrast detection (Dawson et al., 1980). Melanoma (5% of skin cancers) is the most aggressive type of skin cancer, being associated with approximately 75% of skin cancer-related deaths (Karakousis and Czerniecki, 2011). PAI has, therefore, been utilized for the imaging of melanoma in subcutaneous mouse tumor models (Oh et al., 2006; Zhang et al., 2006; Zhou et al., 2014) and for the screening of circulating metastatic melanoma cells, based on cell melanin content detection (Galanzha et al., 2009). It has also successfully been applied to the detection of melanoma metastases in extracted sentinel lymph nodes from animals (McCormack et al., 2009) and human patients with cutaneous melanoma (Grootendorst et al., 2012; Langhout et al., 2014).

The main complication from melanoma is the development of brain metastases which impair brain function by dislodging or killing neurons, thereby inducing cerebral edema. This results in a decline in neurocognitive performance and in intracranial pressure leading to a major cause of death in patients with brain malignancies (Klumpp et al., 2016). The prognosis for such patients is thus very poor, with a median survival rate of 4 to 5 months (Davies et al., 2011). Thus, major improvements in brain metastases detection and subsequent therapeutic management remain critical.

In vivo biological characterization of melanoma brain metastases in experimental models represents a key step to developing new research strategies to improve diagnostic, therapy, and early assessment of treatment efficacy of these tumors in humans.

This study aimed to assess, for the first time, the input of spectroscopic PAI for non-invasive examination of melanoma brain metastases in an orthotopic mouse model and further focused on spatial analyses within the tumor tissue.

2. Material & methods

2.1. In vitro photoacoustic imaging

B16F10 cells (murine skin melanoma) were cultured in DMEM medium supplemented with 10% heat-inactivated Fetal Bovine Serum in humidified atmosphere with 5% CO_2. At 60% confluency, cells were washed twice with 1× PBS before being resuspended in trypsin. After centrifugation at 250*g* for 5 min, cells were suspended in cold 1× PBS and living cells were counted on a Malassez cell after Trypan blue coloration. Cylindrical phantoms were prepared with agarose incorporating titan dioxide and Indian ink in order to mimic brain tissue absorbing (μ_a = 0.2 cm^{-1}) and scattering (μ_s' = 10 cm^{-1}) features. A 1 mm diameter tubing was placed at a 5 mm depth to create a tunnel were cells suspension could be placed. Various concentrations of B16F10 cells in DMEM (0; 6.25×10^3; 1.25×10^4; 2.5×10^4; 5.0×10^4 and 1.0×10^5 cells/μL) were inserted into a phantom (n = 3 per condition) and were submitted to spectroscopic PAI (vevo®LAZR, Fujifilm, Visualsonics Inc., Canada) from 680 to 970 nm with a 1 nm step. Spectral unmixing analyses were performed (Vevo®Lab software according to Luke et al. (2013)) and melanin quantification were made in a 0.1 mm^3 volume of interest corresponding to a slice of the phantom tunnel.

2.2. Brain metastasis animal model

All animal experiments procedures were approved by the animal ethics committee and received the authorization of the French Ministry of Higher Education and Research (reference #4937-2016041209236318 v6).

Female 6 weeks old, NMRI nude mice (Janvier, Le Genest-Isle, France) were implanted with B16F10 cells directly into the brain (n = 6). Anesthetized animals (isoflurane/air 4% for induction and 1.5% thereafter) were shaved and placed on a stereotaxic frame. A subcutaneous injection of 50 μL bupivacaïne (5 mg/mL) was administered locally before the scalp was open to expose the skull. Skull sutures were revealed by gently removing all the membranes located on the external face of the skull. The point of injection was marked on the surface of the cranium (bregma 0, mediolateral 2 mm, dorso-ventral 3 mm) in order to drill a hole and insert the Hamilton syringe. After insertion of the Hamilton syringe into the striatum, the cells suspension (1.0×10^5 cells in 5 μL) was slowly pumped in (5 μL/min). The opening was then stoppered with Horsley wax and the skin sutured.

2.3. In vivo ultrasound and photoacoustic imaging

Ultrasound and PAI were performed with the Vevo®LAZR system (Fujifilm, Visualsonics Inc. Canada) using the LZ201 transducer (256 elements linear array; 15 MHz center frequency, [9–18 MHz bandwith], 100 μm axial resolution; 220 μm lateral resolution; 30 × 30 mm^2 image size) with the addition of a brain-dedicated illumination jacket concentrating the excitation light on a 10 mm-wide slice (Fig. S1). All imaging experiments were conducted under gaseous anesthesia (isoflurane/air 4% for induction and 1.5% thereafter).

3D B-mode, Color Doppler, oxy-hemo (750 and 850 nm) and spectroscopic (680; 710; 728; 894; 924 nm) scans were performed on the entire mice heads. For data analysis, tumor volumes were obtained by manually drawing Volumes of Interest (VOIs) on 3D B-mode images. Total hemoglobin content (HbT) and oxygen saturation rate (StO_2) were calculated from oxy-hemo data (Vevo®Lab software according to Wang et al. (2006)). Melanin, oxy- and deoxy-hemoglobin content were estimated by spectral unmixing analyses of spectroscopic data (Vevo®Lab software according to Luke et al. (2013)).

In addition, HbT and StO_2 were calculated in several sub-volumes of interest along the longitudinal axis of the tumors (Fig. 4A) from the tumors periphery (slices #1 and #5) to the tumors core (slice #3), with 2 intermediate positions (slices #2 and #4).

2.4. Statistical analysis

Statistical analyses were performed using GraphPad Prism version 6 (GraphPad software, San Diego, California USA) and Mann & Withney or One-Way ANOVA tests were applied (*p-value < 0.05; **p-value < 0.01; ***p-value < 0.001; ****p-value < 0.0001).

2.5. Ex vivo brain imaging and histology

Anesthetized mice were euthanized through a 30 μL intracardiac injection of Dolethal (Vetoquinol, France) and were perfused by 4% formaldehyde for tissue fixation. Harvested brains were then placed in a water bowl for *ex vivo* spectroscopic PAI (680; 710; 728; 894; 924 nm), melanin content being estimated by spectral unmixing analyses (Vevo®Lab software). Finally, brains were frozen in OCT Tissue-Tek ™ (Sakura®, Torrance, U.S.A.) and cut in 7 μm thick slices using a cryostat. Slices were stained with hematoxylin and eosin and entirely scanned (mosaic) by Axioimageur M2 Microscope (Zeiss) under 5× magnification.

3. Results

3.1. Melanoma cells photoacoustic signal in phantoms

B16F10 Melanoma cells displayed a specific photoacoustic spectrum that can be clearly separated from those of oxy- and deoxyhemoglobin (Fig. 1A). When inserted 5 mm deep into a phantom with brain tissue absorbing and scattering features, B16F10 cells were clearly detectable, and after spectral unmixing analyses, the photoacoustic signal was shown to be linearly correlated to the cell concentration (R^2 = 0.97). The detection limit in these experimental conditions was found to be