Published in final edited form as:

Ann Surg Oncol. 2014 May ; 21(5): 1435–1440. doi:10.1245/s10434-013-3222-0.

Hypoxia in Melanoma: Using Optical Spectroscopy and EF5 to Assess Tumor Oxygenation Before and During Regional Chemotherapy for Melanoma

Paul J. Speicher, MD¹, Georgia M. Beasley, MD, MHS¹, Belly Jiang, BS², Michael E. Lidsky, MD¹, Gregory M. Palmer, PhD³, Peter M. Scarbrough, PhD³, Paul J. Mosca, MD, PhD^{1,4}, Mark W. Dewhirst, DVM, PhD³, and Douglas S. Tyler, MD^{1,4}

¹Department of Surgery, Duke University Medical Center, Durham, NC

²Duke University School of Medicine, Durham, NC

³Department of Radiation Oncology, Duke University Medical Center. Durham, NC

⁴Division of Surgical Oncology, Duke University Medical Center, Durham, NC

Abstract

Background—There is increasing evidence that tumor hypoxia plays a significant role in the chemoresistance of melanoma, but to our knowledge, real-time tumor oxygenation during isolated limb infusion (ILI) has not been studied. We sought to demonstrate the feasibility of measuring real-time alterations in tissue oxygenation.

Methods—Consecutive patients with histologically confirmed in-transit melanoma were enrolled onto a prospective single-arm pilot study and administered the hypoxia marker drug EF5. All patients were treated with ILI. Optical spectroscopy readings were obtained at three locations: two discrete target lesions and one normal skin control. Measurements were taken at 11 predefined time points during ILI.

Results—A total of six patients were enrolled onto this pilot study. Intratumor and normal skin optical spectroscopy readings were found to have discrete inflection points throughout the duration of therapy, corresponding with established time points. Baseline hypoxia as measured by both optical spectroscopy and EF5 immunofluorescence was variable, but on the basis of optical spectra, tumors appeared to become more hypoxic compared to normal skin after tourniquet application. The optical hypoxia signature was variable between patients while hemoglobin absorption increased.

Conclusions—To our knowledge, this is the first use of real-time optical spectroscopy to evaluate oxygenation and perfusion within melanoma lesions during regional chemotherapy. We report our development of this new noninvasive means of assessing tumor vascular function,

which has the potential to be a powerful tool for noninvasive examination of the melanoma tumor microenvironment.

Although a number of promising advances have recently been made using targeted therapy approaches, melanoma chemoresistance continues to represent a major hurdle in the treatment of this malignancy.¹⁻³ Melanoma has long been thought to have intrinsic resistance to chemotherapy as a result of expression of drug-resistance proteins, but there is increasing evidence that these tumors possess altered vascular pathophysiology, which may act to limit drug delivery into tumor cells.⁴ Regional treatment strategies for in-transit melanoma provide for a unique platform in oncology to develop a better understanding of how the tumor microenvironment affects chemotherapeutic drug delivery to these multifocal tumor deposits found between the site of the primary lesion and its regional draining lymph node basin. Current management of in-transit disease often employs regional chemotherapy in the form of isolated limb infusion (ILI) or isolated limb perfusion (ILP). These strategies are similar in concept, allowing for directed drug delivery and sequential tumor biopsies. but whereas ILP is performed in an oxygenated circuit, ILI is performed under profoundly hypoxic conditions.

Optical spectroscopy is an appealing method for non-invasively characterizing tissue oxygenation and blood flow. utilizing diffuse reflectance and fluorescence spectroscopies. The tissue is illuminated with visible and near-infrared light, and the optical features of the tissue are described on the basis of the measured absorption and scattering properties, which are determined primarily by oxygenated and deoxygenated hemoglobin. A number of preclinical studies have demonstrated that optical spectroscopy has the ability to noninvasively characterize changes in tumor oxygenation and physiology.⁵⁻¹⁰ Clinically, optical spectroscopy has been utilized in a number of areas, particularly in assessing the tumor biology of breast cancer and in predicting an individual's response to treatment.¹¹⁻¹⁴ Although melanoma lesions have not been well studied using this approach, some work has been done in the discrimination of early melanoma from common and dysplastic nevi.¹⁵ We believe that optical spectroscopy has potential to be a powerful tool for predicting individual treatment responses in patients with melanoma, particularly when utilized during regional therapy.

The objective of the current study was to demonstrate the feasibility of measuring real-time alterations in tissue oxygenation in advanced extremity melanoma. We define a technique for measuring tissue hypoxia variations in intransit lesions before and during an isolated regional limb infusion procedure.

METHODS

This study was approved by the Institutional Review Board at Duke University as part of a pilot study investigating the role of the HRHV axis in the tumor microenvironment of patients undergoing regional chemotherapy for in-transit melanoma. Patients with histologically confirmed in-transit melanoma were identified through the melanoma multidisciplinary clinics at Duke University Medical Center and were enrolled in this prospective nonrandomized single-arm study. Inclusion criteria consisted of all patients 18

years of age and older with histologically confirmed AJCC Stage IIIB/IIIC/IV extremity melanoma scheduled to undergo a regional chemotherapy procedure. Patients who were pregnant, had an IV dye allergy, or a history of any previous malignancy treated with radiotherapy and/or chemotherapy were excluded. All study patients were treated with ILI utilizing standard-of-care melphalan.

As part of the study protocol, patients were all given intravenous infusions of the hypoxia marker drug EF5 for future histologic comparisons. EF5 is a 2-nitroimidazole based on related radiosensitizing drugs, but synthesized with the primary purpose of detecting hypoxic cells.¹⁶ The drug is metabolized and retained under hypoxic cellular conditions, has maximum sensitivity at low oxygen levels and has been demonstrated to provide hypoxia measurements at the single-cell level using standard immunofluorescence microscopy techniques.^{17,18} The optical spectroscopy equipment used in this study is not available commercially, and was designed and assembled at our institution. Custom-made fiber optic probes were manufactured, and the spectrometer consisted of a 450 W xenon arc lamp, imaging spectrograph, double excitation monochromator, and Peltier-cooled CCD (Fig. 1).

Optical spectroscopy readings were obtained in the operating room by placing three separate fiber optic probes at three distinct anatomic locations: two on in-transit target lesions and one on adjacent normal skin to act as a control. The probes were placed in direct contact with the lesions or skin, and secured in place with a small transparent dressing (Fig. 2). This is done noninvasively and without damage to the skin or tumor lesion of interest. Because of limitations regarding depth of visible light penetration into the tumor, superficial lesions are preferred to subcutaneous nodules if available. A piece of opaque gauze dressing was then applied on top, so as to prevent ambient light exposure from interfering with the measurements. To further facilitate intra- and inter-patient comparisons, we established a standardized timeline for our measurements at 11 predefined time points during ILI (Fig. 3). We defined these times on the basis of important events during the course of a typical ILI procedure, which were points when significant interventions or physiologic changes were expected to occur. Specifically, the time points were defined as: baseline, pre-tourniquet, tourniquet application, papaverine introduction, immediately before melphalan administration, 1 minute into recirculation, 25 min into recirculation, 30 min into recirculation, postwashout, tourniquet release, and 5 min after reperfusion.

Once the spectroscopic information was acquired at each time point, optical hypoxia signatures were studied by looking at ratios of wavelengths strongly influenced by tissue hypoxia. Specifically, the ratio of 797:758 nm emission was considered, which corresponds to an isosbestic point where oxygenated and deoxygenated hemoglobin absorb equally (797 nm), normalized to a wavelength where deoxyhemoglobin has an absorption peak (758 nm). The use of an isosbestic point for analysis controls for changes in blood volume; thus, an increase in the wavelength ratio would be indicative of increased de-oxyhemoglobin concentration due to hypoxia or pooled deoxygenated blood. To quantify tissue optical properties, we plan to use an inverse Monte Carlo model developed and validated by our group and reported previously.^{19,20}

RESULTS

Between July 2012 and January 2013, 13 patients diagnosed with in-transit melanoma underwent ILI at our institution. Of these, seven met study inclusion criteria. One patient refused participation in the study and received a standard-of-care ILI with melphalan but without optical spectroscopy or EF5 marker drug. Of the six remaining patients enrolled in the study, median patient age was 65 (range 63-75). The interval from initial melanoma diagnosis to study participation and ILI ranged from 4 months to 11 years, and mean Breslow thickness of the primary lesion was 1.43 mm. Five patients (83.3 %) had AJCC stage IIIC malignant melanoma at the time of study enrollment, and the remaining patient was designated stage IIIB as a result of lack of nodal involvement.

Optical spectroscopy readings of tumor and skin controls were found to have discrete stepwise inflection points throughout the duration of therapy, corresponding well with time points defined a priori in our timeline (Figs. 3, 4). Baseline measures of the hypoxia signature revealed variability both between patients and among lesions within patients. There was also significant variability in baseline hypoxia in the tumor microenvironment as characterized using immunofluorescence microscopy of the intravenously administered hypoxia marker drug EF5 (Fig. 5). Deoxyhemoglobin levels as demonstrated by optical spectroscopy rose abruptly upon tourniquet application and drug circulation, consistent with past observations that ischemia develops rapidly upon isolation of the limb from the systemic circulation. Interestingly, our preliminary results demonstrate that tumors appear to respond differently to tourniquet application as compared with in-field normal skin controls. In many cases, tumors developed rapid and profound hypoxia, whereas normal skin control measurements had more modest elevations in de-oxyhemoglobin throughout the procedure. Upon administration of papaverine into the limb circuit, there appeared to be a slight shouldering of the spectroscopy curve, possibly a result of superficial vasodilation leading to a transient decrease in local hypoxia of the skin and subcutaneous tissues. Upon release of the tourniquet, de-oxyhemoglobin levels fell sharply, consistent with rapid reperfusion of the limb. Of note, regardless of differences in tumor and skin response to the tourniquet-induced hypoxia, during reperfusion, the probe measurements rapidly returned to their baseline levels and maintained their pretreatment relationships to each other.

Because of the limited sample size of this pilot study, no formal statistical comparisons can currently be drawn with respect to optical spectroscopy characteristics or patient outcomes. Although the patterns of hypoxia were similar among patients and between lesions, peak deoxyhemoglobin concentrations were variable between patients. For all patients, total hemoglobin was noted to increase at the surface probe positions during ILI.

DISCUSSION

Optical spectroscopy represents a novel tool for noninvasively assessing real-time changes in the tumor microenvironment of melanoma, specifically deoxyhemoglobin concentration and tumor blood volume. Using predefined time points, we were able to demonstrate that tumor hypoxia and blood volume are measurable at baseline and have an ensuing clear relationship with clinically relevant intervals throughout the procedure. We also demonstrate

that total hemoglobin increases at our points of measurement during ILI, suggesting that superficial tissue perfusion, and possibly drug delivery, is increased during this time. To our knowledge, this is the first use of in vivo optical spectroscopy to evaluate oxygenation and perfusion in melanoma lesions during regional chemotherapy. We report our use of this new noninvasive means of interrogating tumor vascular function as part of a pilot study examining the role of tumor hypoxia in patients with in-transit disease. On the basis of our preliminary findings, we believe that optical spectroscopy has potential to be a powerful tool for noninvasive examination of the melanoma tumor microenvironment.

Hypoxia is thought to play a significant role in the tumor response to both radiotherapy and chemotherapy across numerous solid tumors. The extent of hypoxia present in the tumor microenvironment of melanoma remains poorly defined, and our baseline measurements of hypoxia before ILI are highly relevant to our understanding and characterization of this. Chronic low oxygen tension has a significant effect on tumor-specific immune response. As part of this, tumor hypoxia has been demonstrated to modulate the differentiation and function of immune cells, specifically through recruitment of tumor-associated macrophages and regulatory T cells.²¹⁻²³ Activation of HIF-1 α may inhibit tumor-specific T-cell response, and baseline tumor hypoxia may therefore protect against a cytotoxic immune response, with implications for tumor progression and response.²⁴

Furthermore, ILI induces profound hypoxia in the infusion circuit, and although it is unknown how these hypoxic and acidic conditions might affect the tumor microenvironment, this may be important in augmenting the cytotoxicity of melphalan.²⁵⁻²⁷ Early in vitro studies demonstrated that the combination of low oxygen tension and the consequent acidic pH found under hypoxic conditions led to an increase in the cytotoxic effect of melphalan on tumor cells.²⁸ Although it has not been demonstrated that this effect is preserved in vivo during ILIs, there is nonetheless a theoretical basis for this concept. Having a noninvasive means of characterizing hypoxia during the course of regional limb infusion procedures may be important for predicting tumor response.

In applying optical spectroscopy measurements to melanoma, a number of important points must be considered. One of the primary issues is target selection, and our preference is generally to use our clinically defined target lesions as our tumors of interest for optical measurements. These lesions, however, tend to be the largest available, usually to facilitate accurate measurements in assessing postoperative response. Often the lesion is larger than the probe sensor surface, and in such situations, it is our preference to place the probe sensor on the firmest area of the lesion, as the softer areas are presumed to have possibly already undergone necrosis. Although the optical probe is simply affixed to the lesion of interest, other local factors can also be important. As mentioned previously, limitations of light penetrance limit this technique to surface lesions and very superficial deposits. In the setting of limb lymphedema, often as a result of a previous lymph node dissection, measurements may be limited to only the most superficial lesions.

Although the current study is limited by small patient numbers, we hope that this preliminary work will set the stage for larger, more rigorous investigations utilizing optical spectroscopy to assess the tumor microenvironment in melanoma. As part of this, we hope

10 more thoroughly explore the relationship between hypoxia as measured by optical spectroscopy and by immunofluorescence microscopy using the hypoxia marker drug EF5. By combining optical spectroscopy with hypoxia immunofluorescence in future studies, we will be able to more accurately characterize the hypoxic distribution in the tumor microenvironment and validate our noninvasive means of assessing overall tumor hypoxia and perfusion. Additionally, the results of this study have focused solely on tumor interrogation during the profoundly hypoxic conditions of ILI, but in future efforts we plan to apply this same technology to well-oxygenated ILPs. Further studies are needed to fully develop this new means of interrogating tumor vascular function, with the eventual goal of guiding treatment and predicting individual treatment responses among patients with advanced stage extremity melanoma.

Acknowledgments

This work was supported by NIH RO1-CA40355, NIH 5T32CA093245-10, and the Melanoma Research Alliance.

REFERENCES

1. Chapman PB, Hauschild A, Roben C, et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med*. 2011; 364:2507–16. [PubMed: 21639808]
2. Flaherty KT, Robert C, Hersey P, et al. Improved survival with MEK inhibition in BRAF-mutated melanoma. *N Engl J Med*. 2012; 367:107–14.
3. Sosman JA, Kim KB, Schuchter L, et al. Survival in BRAF V600-mutant advanced melanoma treated with vemurafenib. *N Engl J Med*. 2012; 366:707–14.
4. Dewhirst MW, Cao Y, Moeller B. Cycling hypoxia and free radicals regulate angiogenesis and radiotherapy response. *Nat Rev Cancer*. 2008; 8:425–37.
5. Conover DL, Fenton BM, Foster TH, Hull EL. An evaluation of near infrared spectroscopy and cryospectrophotometry estimates of haemoglobin oxygen saturation in a rodent mammary tumour model. *Phys Med Biol*. 2000; 45:2685–700.
6. Finlay JC, Foster TH. Hemoglobin oxygen saturations in phantoms and in vivo from measurements of steady-state diffuse reflectance at a single, short source-detector separation. *Med Phys*. 2004; 31:1949. [PubMed: 15305445]
7. Kim JG, Zhao D, Song Y, Constantinescu A, Mason RP, Liu H. Interplay of tumor vascular oxygenation and tumor pO₂ observed using near-infrared spectroscopy, an oxygen needle electrode, and ¹⁹F MR pO₂ mapping. *J Biomed Opt*. 2003; 8:53–62. [PubMed: 12542380]
8. Liu H, Gu Y, Kim JG, Mason RP. Near-infrared spectroscopy and imaging of tumor vascular oxygenation. *Methods Enzymol*. 2004; 386:349–78. [PubMed: 15120261]
9. Palmer GM, Viola RJ, Schroeder T, Yarmolenko PS, Dewhirst MW, Ramanujam N. Quantitative diffuse reflectance and fluorescence spectroscopy: tool to monitor tumor physiology in vivo. *J Biomed Opt*. 2009; 14:024010.
10. Vishwanath K, Yuan H, Barry WT, Dewhirst MW, Ramanujam N. Using optical spectroscopy to longitudinally monitor physiological changes within solid tumours. *Neoplasia*. 2009; 11:889–900. [PubMed: 19724683]
11. Brown JQ, Wilke LG, Geradts J, Kennedy S, Palmer GM, Ramanujam N. Quantitative optical spectroscopy: a robust tool for direct measurement of breast cancer vascular oxygenation and total hemoglobin content in vivo. *Cancer Res*. 2009; 69:2919–26. [PubMed: 19293184]
12. Cerussi A, Hsiang D, Shah N, et al. Predicting response to breast cancer neoadjuvant chemotherapy using diffuse optical spectroscopy. *Proc: Natl Acad Sci USA*. 2007; 104:4014–9. [PubMed: 17360469]
13. Cerussi AE, Tanamai VW, Hsiang D, Butler J, Mehta RS, Tromberg BJ. Diffuse optical spectroscopic imaging correlates with final pathological response in breast cancer neoadjuvant chemotherapy. *Philos Trans A Math Phys Eng Sci*. 2011; 369:4512–30. [PubMed: 22006904]

14. Ueda S, Roblyer D, Cerussi A, et al. Baseline tumor oxygen saturation correlates with a pathologic complete response in breast cancer patients undergoing neoadjuvant chemotherapy. *Cancer Res.* 2012; 72:4318–28. [PubMed: 22777823]
15. Murphy BW, Webster RJ, Quirk CJ, Clay CD, Heenan PJ, Sampson DD. Toward the discrimination of early melanoma from common and dysplastic nevus using fiber optic diffuse reflectance spectroscopy. *J Biomed Opt.* 2005; 10:1–9.
16. Koch CJ, Hahn SM, Rockwell K, Covey JM, McKenna WG, Evans SM. Pharmacokinetics of EF5 [2-(2-nitro-1-H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl) acetamide] in human patients: implications for hypoxia measurements in vivo by 2-nitroimidazoles. *Cancer Chemother Pharmacol.* 2001; 48:177–87. [PubMed: 11592338]
17. Jenkins WT, Evans SM, Koch CJ. Hypoxia and necrosis in rat 9L glioma and Morris 7777 hepatoma tumors: comparative measurements using EF5 binding and the Eppendorf needle electrode. *Int J Radiat Oncol Biol Phys.* 2000; 46:1005–17.
18. Koch CJ. Measurement of absolute oxygen levels in cells and tissues using oxygen sensors and 2-nitroimidazole EF5. *Methods Enzymol.* 2002; 352:3–31. [PubMed: 12125356]
19. Palmer GM, Ramanujam N. Monte Carlo-based inverse model for calculating tissue optical properties. Part I: theory and validation on synthetic phantoms. *Appl opt.* 2006; 45:1062–71. [PubMed: 16512550]
20. Palmer GM, Zhu C, Breslin TM, Xu F, Gilchrist KW, Ramanujam N. Monte Carlo-based inverse model for calculating tissue optical properties. Part II: application to breast cancer diagnosis. *Appl opt.* 2006; 45:1072–8. [PubMed: 16512551]
21. Dang EV, Barbi J, Yang HY, et al. Control of T(H)17r(reg) balance by hypoxia-inducible factor 1. *Cell.* 2011; 146:772–84. [PubMed: 21871655]
22. Du R, Lu KV, Petritsch C, et al. HIF1 α induces the recruitment of bone marrow-derived vascular modulatory cells to regulate tumor angiogenesis and invasion. *Cancer Cell.* 2008; 13:206–20. [PubMed: 18328425]
23. Palazón A, Aragonés J, Morales-Kastresana A, de Landázuri MO, Melero I. Molecular pathways: hypoxia response in immune cells fighting or promoting cancer. *Clin Cancer Res.* 2012; 18:1207–13. [PubMed: 22205687]
24. Sitkovsky M, Lukashev D. Regulation of immune cells by local-tissue oxygen tension: HIF1 α and adenosine receptors. *Nat Rev Immunol.* 2005; 5:712–21. [PubMed: 16110315]
25. Gerweck LE, Vijayappa S, Kozin S. Tumor pH controls the in vivo efficacy of weak acid and base chemotherapeutics. *Mol Cell Cancer Ther.* 2006; 5:1275–9.
26. Kroon HM, Moncrieff M, Kam PCa, Thompson JF. Outcomes following isolated limb infusion for melanoma. A 14-year experience. *Ann Surg Oncol.* 2008; 15:3003–13. [PubMed: 18509706]
27. Prescott DM, Charles HC, Poulson IM, et al. The relationship between intracellular and extracellular pH in spontaneous canine tumors. *Clin Cancer Res.* 2000; 6:2501–5. [PubMed: 10873105]
28. Siemann DW, Chapman M, Beikirch A. Effects of oxygenation and pH on tumor cell response to alkylating chemotherapy. *Int J Radiat Oncol Biol Phys.* 1991; 20:287–9.

FIG. 1.
Custom-made optical spectrometer consisting of a 450 W xenon arc lamp (*arrowhead*),
imaging spectrograph, double excitation monochromator and Peltier-cooled CCD (*arrow*).
fiber optic switch and cables (*asterisk*), and laptop computer

FIG. 2.
Optical probes (*arrows*) attached to fiber optic cables (*arrowheads*), shown secured to skin with clear adhesive dressing, before being covered with towels to prevent ambient light noise

FIG. 3. Timeline of a typical ILI, with time points T1 through T11 indicated for standardization of optical spectroscopy measurements. Biopsy time points for EF5 tissue collection are also indicated

FIG. 4. Representative optical spectroscopy results, showing the ratio of 797:758 nm diffuse reflectance, plotted against procedure progression. The tumor hypoxia signature (*blue* and *green*) appears to be more pronounced than normal skin (*red*) in response to limb ischemia during ILI

FIG. 5. Representative punch biopsy samples of in-transit melanoma lesions using immunofluorescence microscopy at $\times 5$ magnification. Hypoxia (anti-EF5 antibody) is shown in *red*, endothelium (anti-CD31) in *green*, and cell nuclei (DAPI) in *blue*. There is considerable variation between patients with respect to baseline hypoxia (*red*), which appears to be inversely correlated with tumor vascularization as shown by CD-31 (*green*)