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Optical Spectrsocopy of Murine breast tumor to distinguish indolent from aggressive disease

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Optical Spectroscopy of Murine Breast Tumors to Distinguish Indolent from
Aggressive Disease

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Abstract

Breast cancer accounts for 30% of all cancer. Metastasis is the primary cause of death among breast cancer patients. Additionally, current molecular profiling methods such as Oncotype DX, which are expensive and not widely available at all clinical facilities, only determine the risk of recurrence after treatment. Therefore, there are no current method capable of identifying metastatic patients in advance. As a result, there is an unmet clinical need to develop a cost-effective prognostic to differentiate between indolent and aggressive breast tumors. In this study, we implemented diffuse reflectance spectroscopy (DRS) system to evaluate functional changes in tumor xenografts originated from four breast cancer cell lines with variable metastatic potential. The murine mammary cell lines 4T1, 4T07, 168FARN, and 67NR were injected into the flanks of mice to grow tumor xenografts. In addition, we used CRISPR/Cas-9 to delete TWIST- a gene known to promote tumor metastasis - and generate an indolent version of 4T1 (4T1-TWIST KO) that was also grown as xenografts. We collected optical data from these tumors bearing animals and using a look-up table inverse model, we determined vascular oxygen saturation (SO2), total hemoglobin concentration (cHb), and tissue light scattering at four different tumor volumes. Our preliminary data shows functional differences between indolent and aggressive tumors that can be further investigated in human cell line and patient-derived tumors.

Introduction

Breast cancer is a frequent malignancy with high mortality due to the effects generated by the primary tumor and its high capacity to produce distant metastases [1]. 30% of breast cancer patients are diagnosed at an early stage of the disease, and they are likely to experience recurrence of the disease with distant metastasis, which accounts for 90% of deaths among breast cancer [2]. Clinically, doctors rely on anatomical markers, such as tumor stage and lymph node status for decisions related to neoadjuvant chemotherapy, and current methods to assess chemotherapy benefits such as Oncotype DX, based on molecular profiling, are only capable of determining risk of recurrence after treatment [3]. Additionally, Oncotype DX is not available at all clinical facilities, it is highly expensive, and its application is limited by tumor size and established tumor receptors [3,4]. So, there is still an unmet clinical need to differentiate between indolent and aggressive disease.

As a result, the development of a cost-effective prognostic tool that can be implemented to evaluate metastatic potential could significantly aid to the treatment of breast cancer patients [5]. So, it is desirable to identify differences in tumor biology and physiology that come from the metastatic nature of the primary tumor. In this context, optical spectroscopy techniques are highly attractive due to their capacity to provide insights on tumor vascularity, tissue scattering, and oxygenation.

To illustrate, Diffuse reflectance spectroscopy (DRS), which is a widely used optical spectroscopy technique, allows the characterization of the tumor microenvironment through light that has undergone multiple scattering events within a sample of interest [6]. DRS have been proven to assess tumor vascularity and oxygenation because the spectra diffusely reflected from tissue, depends on the scattering and absorption properties of the tumor [7]. From the collected

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light, the reflectance spectrum can be generated, and tissue scattering, hemoglobin concentration (cHb), and oxygen saturation (sO2) can be determine.

In this study, we implemented a diffuse reflectance spectroscopy (DRS) system to evaluate functional changes in tumor xenografts that originated from five breast cancer cell lines with variable metastatic potential. The isogenic murine mammary cell lines, 4T1, 4T07, 168FARN, and 67NR (in descending metastatic potential), were derived from a breast tumor growing in a Balb/c mouse [8]. All of the murine mammary cell lines are capable of primary tumor formation; however, 4T1, 4T07, and 168FARN are the only ones capable of intravasation. 4T1 and 4T07 can extravasate, and only 4T1 is capable of metastatic growth. Studies on these cell lines have shown that TWIST plays a significant role in their metastatic potential [9,10]. TWIST, considered a master regulator of metastasis, was profoundly expressed in not only this family of isogenic cell lines but also in several invasive and metastatic human tumors [10]. So, the ability to knock out the TWIST gene can provide information regarding differences between indolent and aggressive breast tumors. Therefore, the aim of our study was to use Optical Spectroscopy to determine functional differences in the primary tumor between metastatic and non-metastatic cell lines.

Materials and Methods

Cell Culture and Tumor Xenograft

The cell lines, 4T1, 4T07, 67NR, and 168FARN, derived from a spontaneous breast tumor growing in a Balb/c mouse, were provided by Dr. Fred Miller (Karmanos Cancer Institute). CRISPR/Cas-9 was used to generate an indolent version of 4T1 by knocking out the TWIST gene (4T1-TWIST KO). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)

with the addition of 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 1% (v/v) nonessential amino acids, and 1% (v/v) penicillin-streptomycin. The cells were cultured in a humidified incubator set to 5% CO_2 and 37°C and passaged when they reached approximately 80% confluence. Cells were used within the first 10 passages for all experiments. The five murine mammary adenocarcinoma cells were injected into the flanks of 10 Balb/c mice each to grow xenografts (n=50). Tumors xenograft were formed by injecting 2 million cells suspended in 1:1 Matrigel.

CRISPR/Cas9 for the deletion of TWIST gene

The sgRNA guide tool provided by the Zhang laboratory (MIT, Cambridge, MA) was used to identify sgRNA to aim at the TWIST gene. The 20-base pair sgRNA was cloned into pCasGuide-EF1a-GFP plasmids through the services of OriGene (Rockville, MD). E. Coli bacteria were used to expand the plasmid, which was isolated utilizing the QIAGEN Plasmid Maxi Kit. Plasmid transfection was achieved by seeding 4T1 cells in a 6-well plate at a concentration of one million cells/well for an incubation time of 24 for hours. 10 μg of plasmid were added to Lipofectamine 3000 to be added to the 4T1 cells. After 24 to 48 hours, a Nikon TiE fluorescence microscope workstation with a ColSnap HQ2 camera was used to detect the signals exhibiting green fluorescent proteins (GFP), indicating cell transfection. A PBS solution was used to suspend the transfected 4T1 cells at a concentration of two million cells per mL, which were subsequently filtered into a FACS tube with a 50 μm filter. The cells were sorted through the FACS Aria III System (BD Biosciences, San Jose, CA). The 5% percent of transfected cells with the highest GFP expression were classified as cells with the greatest CRISPR / Cas9 plasmid concentration. The classified cells were incubated for 7 to 14 days and

the formed cell colonies were cloned into 13 clones to produce a cell population, having the least TWIST expression.

Diffuse Reflectance Spectroscopy

The implemented DRS setup consisted of a tungsten halogen lamp (HL-2000, Ocean Optics; Dunedin, Florida) supplying a fiber optic spectrometer for data acquisition (Flame, Ocean Optics; Dunedin, Florida), and a fiber-optic probe (dia. = 200 μm, NA = 0.22; FiberTechOptica, Ontario, Canada) with source-detector separation of 2.25 mm (figure 1A). The diffusely reflected light was collected by the five peripheral fibers of the fiber optic probe, which surround four source central fibers (figure 1B). DRS spectra acquisition were performed at four different tumor volumes, 50, 100, 150, and 200 mm3, for three specific murine mammary cell lines, 4T1, 4T1-TWIST KO, and 67NR. DRS spectra acquisition for 4T07 and 168FARN were performed at 200 mm^3.

Quantification of tissue optical properties and statistical analysis

Using an empirical lookup table-based inverse model, we determined scattering, total hemoglobin concentration (cHb), and the vascular oxygen saturation (sO$_2$). A nested, two-way analysis of variance (ANOVA) was implemented to determine statistical significant differences in tissue scattering, hemoglobin concentration and vascular oxygenation across the five murine mammary cell lines, 4T1, 4T1, 4T07, 67NR, 168FARN, and 4T1-TWIST KO.
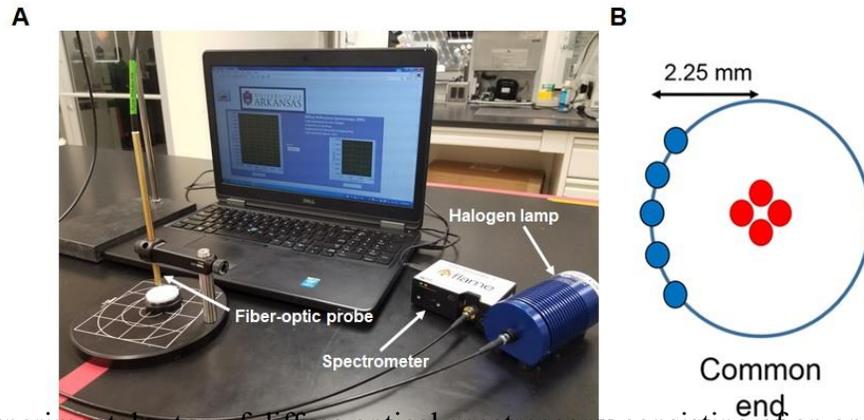
Figure 1. A. Experimental setup of diffuse optical spectroscopy consisting of an optical fiber probe delivering light supplied from a halogen lamp and transferring the reflected light from tissue to a spectrometer. **B.** Representation of the probe common end with a source detention separation of 2.25mm depicting the five peripheral fibers and the four source central fibers.

Results and Discussion

Representative in vivo reflectance, absorption and scattering

Representative DRS spectra of the five murine breast cancer cell lines at 200 mm^3 are shown on figure 2A. The solid black line indicates the *in vivo* measurements, and the solid red lines the LUT model fit. The DRS spectra showed that 4T07 and 168FARN presents have more pronounced hemoglobin band, which is the section of the curve between 540 and 580 nm, indicating high oxygen saturation. In comparison with 4T1 and 67NR, which have a flatter or less pronounce hemoglobin band, indicating lower oxygen saturation. It is also worth noting that 4T1-TWIST KO have a more pronounced hemoglobin band than 4T1. The corresponding dependent absorption coefficients are represented in figure 2B. 4T07, 168FARN, and 4T1-TWIST KO, shown in the absorption plot as a function of wavelength, present the two distinctive peaks between 540 and 580 nm characteristic of the hemoglobin absorption profile. 67NR has the highest absorption levels but with low oxygenation and 4T1 displayed a lower magnitude of the absorption coefficient in comparison to the 4T1-TWIST KO cell line. As shown in figure 2C,

4T07 and 168FARN present the highest scattering coefficients, while the 67NR tumor
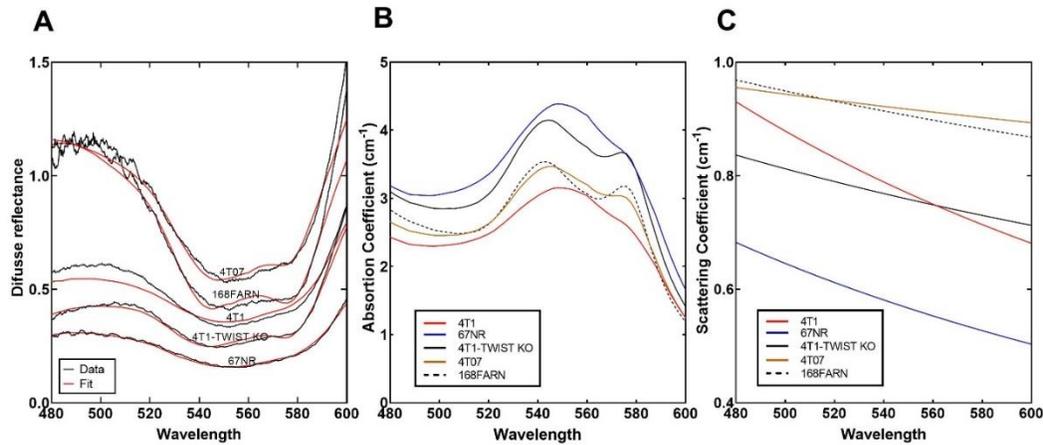
population presents the lowest.

Figure 2. A. Diffuse reflectance spectra (black line) and the corresponding experimental-fit (red line) from representative animals from different groups. **B.** Absorption coefficient as a function of wavelength for the different tumor populations **C.** Scattering coefficient as a function of wavelength for the different tumor populations.

Significant differences in hemoglobin concentration and LIGHT scattering among the studied
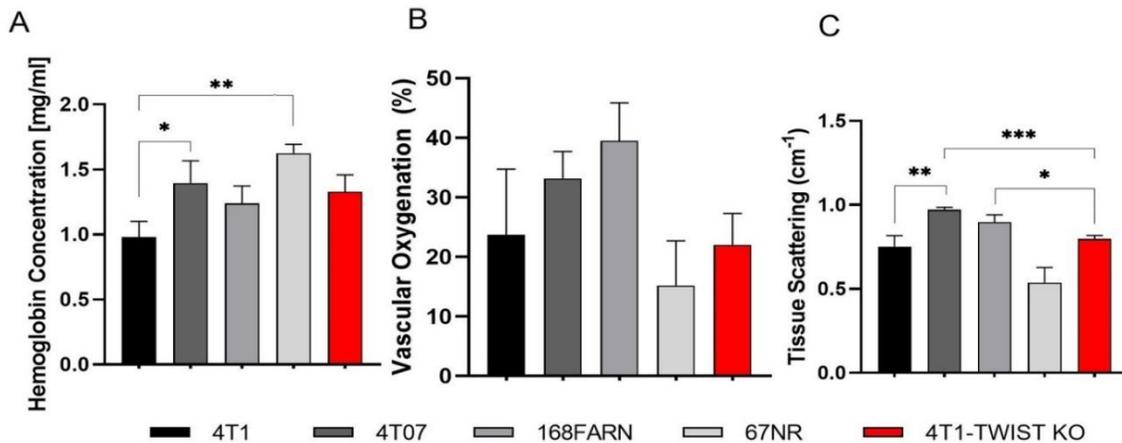
cell lines at 200 mm³

Figure 3 A. Significant differences in total hemoglobin content between 4T1 and 4T07 xenografts and between 4T1 and 67NR at 200 mm³. **B.** Vascular oxygen saturation with no significant differences between the different tumor populations. **C.** Significant Differences were detected between the highly metastatic 4T1 cell line and the 4T07 tumor population and between 4T07 and 4T1- TWIST KO at 200 mm³.

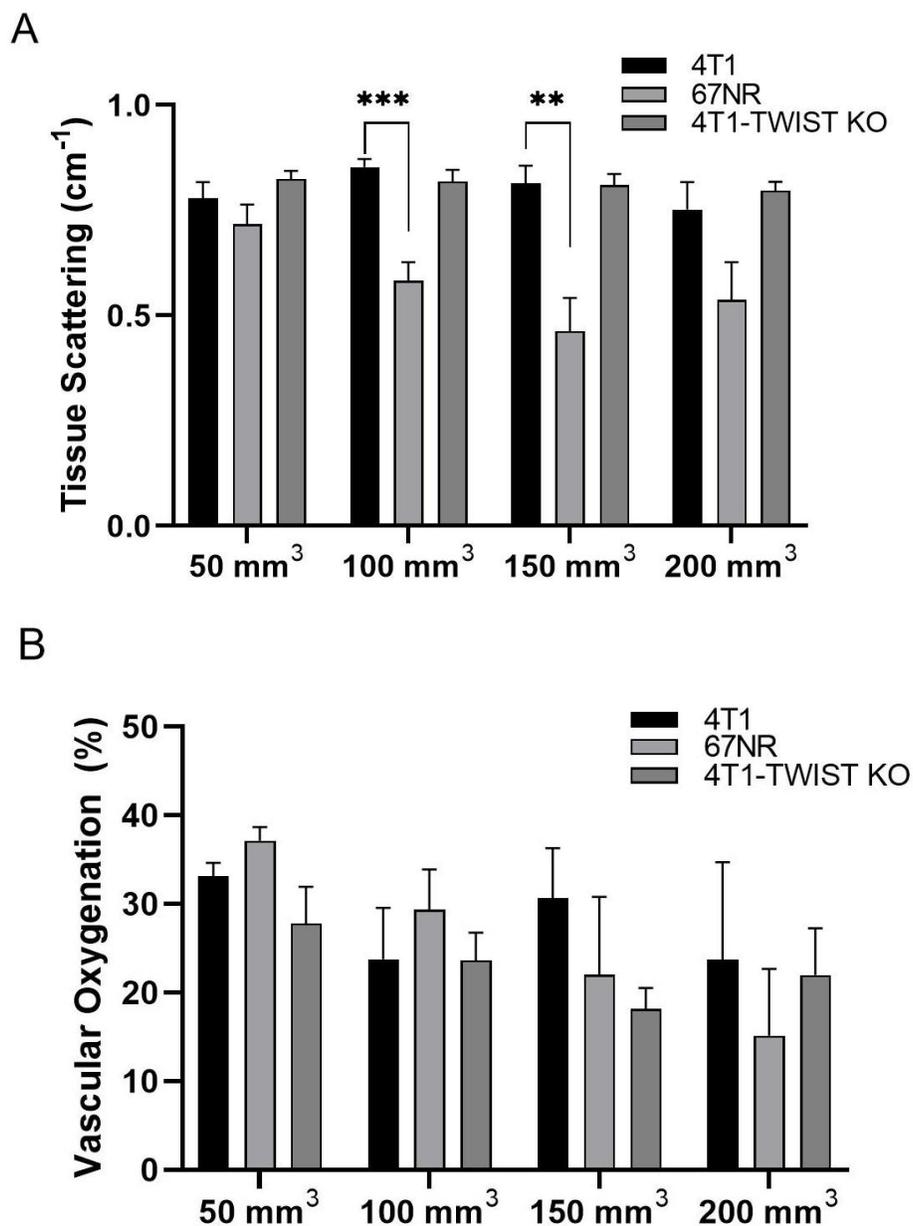*Scattering is significantly greater in the metastatic 4T1 tumors compared with the non-metastatic 67NR tumors*

Figure 4 A. Tissue Scattering as a function of tumor growth reveal there are significant functional differences between the metastatic cell line 4T1 and the non-metastatic cell line 67NR. **B.** Vascular oxygen saturation decreases as tumor volume increases shifting lower and lower in the non-metastatic cell lines 67NR and 4T1-TKO.

There were significant differences in hemoglobin concentration and tissue scattering among the studied cell lines at 200 mm^3. As shown in figure 3A, total hemoglobin concentration was shown to be different between the 4T1 and the 4T07 tumor populations with statistically significant differences between the two groups (p=0.08). Similarly, there were statistically significant differences between 4T1 and 67NR on hemoglobin concentration (p=0.009). It is also noticeable that total hemoglobin concentration has a slight increase as metastatic potential decreases with 4T1 having the lowest levels and 67NR the highest.

Tissue scattering is significantly greater in the metastatic 4T1 tumors compared with the non-metastatic 67NR tumors (figure 4A). Statistical analysis of the scattering intensity data showed a significant difference at 100 mm3 (p<0.001), and at 150 mm3 (p=0.005). It is noticeable that there is a decrease of tissue scattering as tumor volume increases on the 67NR tumor population. No statistically significant interactions on tissue scattering were found between the 4T1 and the 4T1-TWST KO tumor population across the four tumor volumes. The significant difference between 4T1 and 67NR presents the question regarding the source of scattering. Even though we knock down twist in the primary tumor, tissue scattering essentially stays the same between 4T1 and 4T1-TWIST KO and giving that DRS is highly sensitive to collagen, future studies on the primary tumor of the non-metastatic 67NR and the metastatic 4T1 cell lines are required to explain such phenomenon. Additionally, as shown in figure 3C, there were significant differences in tissue scattering between the 4T1 and the 4T07 tumors, between 168FARN and 4T1-TWIST KO tumors, and between 4T07 and 4T1-TWIST KO tumors at 200 mm^3.

Regarding vascular oxygen saturation for each cell line, 4T1,67NR and 4T1-TWIST KO, is represented in figure 4B at the four different tumor volumes. A downtrend on sO$_2$ is observed in the 67NR and the 4T1-TWIST KO tumors. TWIST deletion showed to demote sO$_2$ on the 4T1

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cell line. However, there were no significant differences in sO2 between 4T1 and 4T1-TWIST KO (p>0.05).

Conclusion

Metastatic spread is the primary cause of death among breast cancer patients. Clinically, doctors rely on anatomical markers or highly expensive molecular profiling methods for early detection of metastatic patients. However, a precise classification of metastatic from non-metastatic tumors continues to be a challenge. With non-invasive techniques such as diffuse reflectance spectroscopy, understanding of functional differences can be achieved to differentiate indolent from aggressive disease. In this study, we have shown that diffuse reflectance spectroscopy is sensitive to differences in hemoglobin content between metastatic 4T1 and non-metastatic 67NR tumors. Additionally, our results indicate significant differences in scattering between metastatic (4T1) and non-metastatic tumors (67NR), and a downtrend of vascular oxygen saturation in non-metastatic tumors as tumor volume increases. The preliminary results of this study demonstrate the potential of diffuse reflectance spectroscopy on detecting functional biomarkers that can be implemented in sorting metastatic and non-metastatic tumors.

Acknowledgements

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