Increased metabolic activity detected by FLIM in human breast cancer cells with desmoplastic reaction: a pilot study

Rodrigo de Andrade Natal[1]; Vitor Bianchin Pelegati[2]; Caroline Bondarik[1]; Guilherme Rossi Mendonça[1]; Sophie Françoise Derchain[3]; Carmen Passos Lima[4]; Carlos Lenz Cesar[2]; Luís Otávio Sarian[3]; José Vassallo[1]

Departments of [1]Pathology, [3]Obstetrics and Gynecology, [4]Internal Medicine – Faculty of Medical Sciences; and [2]Optics and Photonics Research Center, Biomedical Lasers Application Laboratory – "Gleb Wataghin" Institute of Physics – State University of Campinas, Campinas, São Paulo, Brazil.

Correspondence to: José Vassallo MD, PhD. Department of Pathology, Faculty of Medical Sciences, State University of Campinas. Rua Tessália Vieira de Camargo, 126, Zip code: 13083-970 – Campinas, São Paulo, Brazil. E-mail: vassallomeister@gmail.com. Phone: +55 19 5521-8987

ABSTRACT

Introduction: In breast cancer (BC), desmoplastic reaction, assembled primarily by fibroblasts, is associated with unfavorable prognosis, but the reason of this fact remains still unclear. In this context, nonlinear optics microscopy, including Fluorescence Lifetime Imaging Microscopy (FLIM), has provided advancement in cellular metabolism research. In this paper, our purpose is to differentiate BC cells metabolism with or without contact to desmoplastic reaction. Formalin fixed, paraffin embedded samples were used at different points of hematoxylin stained sections. **Methodology**: Sections from 14 patients with invasive ductal breast carcinoma were analyzed with FLIM methodology to NAD(P)H and FAD fluorescence lifetime on a Confocal Upright LSM780 NLO device (Carl Zeiss AG, Germany). Quantification of the fluorescence lifetime and fluorescence intensity was evaluated by SPC Image software (Becker &Hickl) and ImageJ (NIH), respectively. Optical redox ratio was calculated by dividing the FAD fluorescence intensity by NAD(P)H fluorescence intensity. Data value for FLIM measurements and fluorescence intensities were calculated using Wilcoxon test; $p < 0.05$ was considered significant. **Results**: BC cells in contact with desmoplastic reaction presented a significantly lower NAD(P)H and FAD fluorescence lifetime. Furthermore, optical redox ratio was also lower in these tumor cells. **Conclusion**: Our results suggest that contact of BC cells with desmoplastic reaction increase their metabolic activity, which might explain the adverse prognosis of cases associated with higher peritumoral desmoplastic reaction.

Key-words: Breast Cancer; Fluorescence lifetime imaging microscopy; Desmoplastic reaction

Advanced Microscopy Techniques IV; and Neurophotonics II, edited by Emmanuel Beaurepaire, Peter T. C. So, Francesco Pavone, Elizabeth M. Hillman, Proc. of SPIE-OSA Vol. 9536, 95360L
 · doi: 10.1117/12.2183442

1. INTRODUCTION

It is known that the extracellular matrix (ECM) can modulate cancer behavior, influencing tumor growth, differentiation and invasion, thus potentially conferring prognostic and predictive information[1]. In breast cancers (BC), there is a scirrhous or desmoplastic reaction caused by interactions between tumor and stromal cells, mainly fibroblasts[2]. In fact, theses fibroblasts can activate BC development and progression through the release of bioactive molecules, like chemokines and growth factors. In addition, these molecules contribute to ECM alterations and remodeling, characteristic of this neoplasm[3,4]. Further, desmoplastic reaction is an important independent recurrence risk marker for breast cancer, and its presence is linked with infiltrative and stellate pattern, concluding that an intense desmoplastic reaction is associated to a poor prognosis[5,6]. However, metabolic alterations related to desmoplastic reaction in BC cells have not yet been elucidated, especially in human BC.

Tumor cells have increased cell metabolism when compared to normal cells, due to their rapid proliferation[7]. This fact is linked with change in the distribution and the relative concentrations of metabolic coenzymes, NAD(P)H and FAD[8-10]. Further, NAD(P)H and FAD are autofluorescence, but they differ with distinct maxima excitation and emission wavelength, so it is possible to isolate each one with optical techniques[11]. The most common method for evaluating cell metabolism is the "optical redox ratio" (ratio between NAD(P)H and FAD fluorescence intensity)[12,13], wherein, low values usually indicate increased metabolic activity[13].

Fluorescence Lifetime Imaging Microscopy (FLIM) is a nonlinear microscopic technique capable of measuring NAD(P)H and FAD lifetime fluorescence[12]. Changes in these lifetime values reflect cell microenvironment alterations, like oxygen, tyrosine and tryptophan concentrations, temperature, and pH[12,14], in summary, cellular metabolism, both *in vivo* and on routinely fixed samples[11,15]. Studies performed with paraffin fixed and unfixed mouse tissue specimens recognized that the fixation process did not significantly impact in the reliability of NAD(P)H and FAD measurements[11,15]. Other reports have also demonstrated that the chemical environment around the fluorophores is somehow preserved after routine histological sample processing[16]. As the fixation and embedding process of tissue samples in our institution is similar to the one described by these authors, it is reasonable to assume that we may compare metabolic states in different specimens even after the fixation procedure. Using this approach, our purpose was to differentiate tumor cell metabolism with or without contact to desmoplastic reaction, in patients with BC, using formalin fixed, paraffin embedded samples, at different points of hematoxylin stained sections.

2. MATERIALS AND METHODS

2.1. Patients and tissues

This is a retrospective study, using formalin fixed paraffin embedded (FFPE) archival tissue specimens from the Department of Pathology of the Faculty of Medical Sciences – State University of Campinas (UNICAMP, São Paulo,

Brazil). Breast cancer specimens were selected from consecutive 14 female patients, who were submitted to primary mastectomy or quadrantectomy. None of these patients had received previous chemotherapy/radiotherapy, nor had they history of other types of cancer. The Research Ethics Committee of the Faculty of Medical Sciences – UNICAMP approved this study (CEP #087/2008).

Four µm sections stained with hematoxylin and eosin staining were reviewed to assess pathological characteristics and to select regions of interest (ROIs), based on: (1) BC cells with desmoplastic reaction (WD), and (2) BC cells without desmoplastic reaction (WoD) (**Figure 1**). The ROIs were transported to the consecutive hematoxylin stained sections to observe fluorescence lifetime and intensity.

2.2. Experimental setup

We used a 40x/1.3 NA oil immersion EC Plan-Neofluar objective on a Confocal Upright LSM780 NLO device (Carl Zeiss AG, Germany) equipped with a Becker & Hickl TCSPC FLIM system. FLIM was excited with a 405 nm diode laser (BDL-405-SMC, Becker & Hickl, Berlin, Germany) with 65 ps pulses and 80 MHz repetition rate. A filter cube with a longpass LP495 nm at 45° splitted the beams in two, one arm with a bandpass BP445±45 nm to capture the NAD(P)H signal and the other with a bandpass BP535±22 nm to capture the FAD signal. Each region with 177 x 177 μm^2 (256 x 256 pixels) was excited for 120s at a rate of approximately $1x10^5$ph/s and detected with the Becker & Hickl PMH-100 detector. The photon count rate did not change during the acquisition process, ensuring that photobleaching did not occur (**Figure 2**).

2.3. Image and statistical analysis

In all 56 images (four images of each case: NAD(P)H autofluorescence, FAD autofluorescence, NAD(P)H-fluorescence lifetime, and FAD- fluorescence lifetime) BC cells were delimited. The fluorescence intensity was determined using the ImageJ software (NIH, USA). "Optical redox ratio" was performed dividing the quantity of FAD fluorescence intensity by the quantity of NAD(P)H fluorescence intensity (1)[12].

$$\text{Optical redox ratio} = \frac{\text{FAD integrated density}}{\text{NAD(P)H integrated density}} \qquad (1)$$

The SPC Image software (Becker & Hickl) was used to analyze the fluorescence lifetime of each metabolic molecules (NAD(P)H and FAD), as described previously by our group[17], with applied binning of 5 x 5. Data are expressed by median values and interquartile range. We compared fluorescence lifetime and "optical redox ratio" measurements using Wilcoxon tests; $p< 0.05$ was considered significant. Statistical analysis was performed using Prism 5.0 (GraphPad Software, La Jolla, CA, USA).

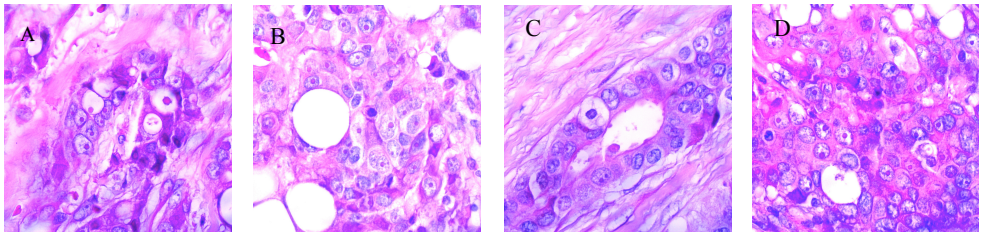

Figure 1: Images A to D feature the histological findings from two distinct patients. Images A and C represent breast cancer (BC) cells with desmoplasia, while images B and D represent BC cells without desmoplasia. Hematoxylin and eosin stained slices (400x).

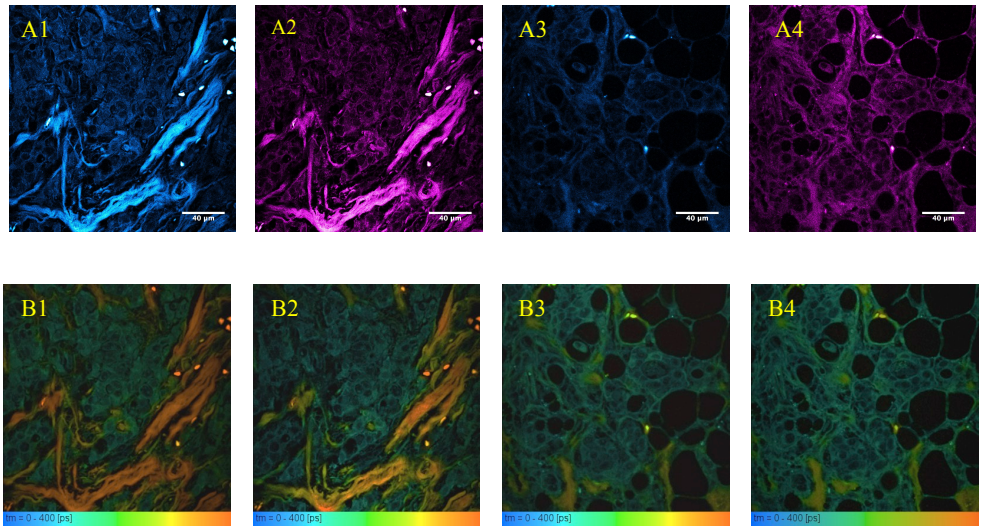

Figure 2: A: fluorescence intensity; B: FLIM image. From left to right: (1) NAD(P)H in breast cancer (BC) cells with desmoplasia (WD); (2) FAD in BC cells WD; (3) NAD(P)H in BC cells without desmoplasia (WoD), and (4) FAD in BC cells WoD.

3. RESULTS

In all 14 patients there were ROIs with BC cells WD and WoD. Further, the fluorescence intensity and lifetime was feasible in all demarcated regions in the hematoxylin stained slices. **Figure 3** shows that BC cells WD had faster NAD(P)H lifetime fluorescence, 293.0 ps (172.7 – 382.4), compared to BC cells WoD, 382.9 ps (370.4 – 154.2) (p= 0.04). FAD lifetime was faster in BC cells WD, 299.0 ps (171.2 – 428.4), when compared to BC cells WoD, 343.5 (236.9 – 535.9) (p= 0.04).

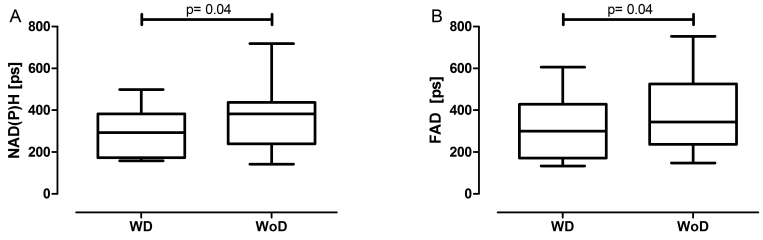

Figure 3: Comparison between BC cells WD and WoD for: (A) NAD(P)H fluorescence lifetime; and (B) FAD fluorescence lifetime. Wilcoxon test was applied with significant p less than 0.05.

Further, **Figure 4** shows that BC cells WD had a decrease of the "optical redox ratio", 1.29 (1.00 – 2.32), as compared to BC cells WoD, 1.89 (1.39 – 2.92) (p= 0.01).

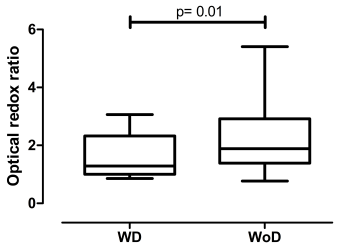

Figure 4: Comparison between "optical redox ratio" in BC cells WD and WoD. Wilcoxon test was applied with significant p less than 0.05.

4. Discussion

In this preliminary study, our results showed that the use of FLIM is feasible in hematoxylin stained sections from routinely FFPE breast cancer samples, obtained from the archives of the Pathology Laboratory. Further, on the same section we observed different fluorescence lifetime patterns, depending on the ROI. The statistical analyzes revealed that BC cells WD had a NAD(P)H and FAD fluorescence lifetime faster than the BC cells WoD. Further, metabolism was higher in the former than in the latter.

In vitro study demonstrated that NAD(P)H has a lower average fluorescence lifetime in highly metastatic variant of cancer cells, when compared to the low metastatic variant, both in rat or human linages[18]. Further, in pancreatic cancer animal model the results were similar, that is, histological high grade pancreatic cancer was associated with a reduction in average fluorescence lifetime in both bound and free NAD(P)H. However, no association was found for the FAD average fluorescence lifetime[12]. In this context, desmoplastic reaction could influence an infiltrative tumor pattern[5,6] by enhancing proliferation and spread of BC cells, as result of increased metabolic activity. Therefore, our

finding that BC cells WD are more metabolically active due to lower NAD(P)H and FAD fluorescence lifetime is supported by previous data. Further, "optical redox ratio" reinforced lower average fluorescence lifetime found in our samples, since the low value of this ratio indicates an increase in metabolic activity.

Despite the evidences presented herein of more aggressive behavior of BC cells in contact with desmoplastic reaction, the limited number of cases did not allow survival analyzes necessary to demonstrate the clinical implications of these findings (e.g., recurrence rate, progression-free survival and global survival). To specifically address the clinical value of these findings, studies with larger number of patients is needed.

In summary, our results using FLIM applied on routinely processed BC sections support the assumption that the non cellular stromal component, as the desmoplastic reaction, could influence cancer cell metabolism, inducing higher activity, and, consequently, more infiltrative and metastatic potential. Recognition of such mechanisms could be the basis of novel therapeutic approaches in the future.

5. REFERENCES

[1] Bergamaschi, A., Tagliabue, E., Sørlie, T., Naume, B., et al., "Extracellular matrix signature identifies breast cancer subgroups with different clinical outcome" J Pathol 214(3), 357-67 (2008).

[2] Al-Adnani, M., Kirrane, J., McGee, J., "Inappropriate production of collagen and prolyl hydroxylase by human breast cancer cells in vivo" Br J Cancer 31(6), 653-60 (1975).

[3] Kalluri, R., Zeisberg, M., "Fibroblasts in cancer" Nat Rev Cancer 6(5), 392-401 (2006).

[4] Orimo, A., Gupta, P., Sgroi, D., Arenzana-Seisdedos, F., et al., "Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion" Cell 121(3), 335-48 (2005).

[5] Ni, Y., Tsang, J., Chan, S., Tse, G., "A novel morphologic-molecular recurrence predictive model refines traditional prognostic tools for invasive breast carcinoma" Ann Surg Oncol 21(9), 2928-33 (2014).

[6] Mujtaba, S., Ni, Y., Tsang, J., Chan, S., et al., "Fibrotic focus in breast carcinomas: relationship with prognostic parameters and biomarkers" Ann Surg Oncol 20(9), 2842-9 (2013).

[7] Gulledge, C., Dewhirst, M., "Tumor oxygenation: a matter of supply and demand" Anticancer Res 16(2), 741-9 (1996).

[8] Heikal, A., "Intracellular coenzymes as natural biomarkers for metabolic activities and mitochondrial anomalies" Biomark Med 4(2), 241-63 (2010).

[9] Zhang, Z., Li, H., Liu, Q., Zhou, L., et al., "Metabolic imaging of tumors using intrinsic and extrinsic fluorescent markers" Biosens Bioelectron 20(3), 643-50 (2004).

[10] Banerjee, S., Bhatt, D., "Histochemical studies on the distribution of certain dehydrogenases in squamous cell carcinoma of cheek" Indian J Cancer 26(1), 21-30 (1989).

[11] Conklin, M., Provenzano, P., Eliceiri, K., Sullivan, R., et al., "Fluorescence lifetime imaging of endogenous fluorophores in histopathology sections reveals differences between normal and tumor epithelium in carcinoma in situ of the breast" Cell Biochem Biophys 53(3), 145-57 (2009).

[12] Skala, M., Riching, K., Gendron-Fitzpatrick, A., Eickhoff, J., et al., "In vivo multiphoton microscopy of NADH and FAD redox states, fluorescence lifetimes, and cellular morphology in precancerous epithelia" Proc Natl Acad Sci U S A 104(49), 19494-9 (2007).

[13] Chance, B., Schoener, B., Oshino, R., Itshak, F., et al., "Oxidation-reduction ratio studies of mitochondria in freeze-trapped samples. NADH and flavoprotein fluorescence signals" J Biol Chem 254(11), 4764-71 (1979).

[14] Suhling, K., French, P., Phillips, D., "Time-resolved fluorescence microscopy" Photochem Photobiol Sci 4(1), 13-22 (2005).

[15] Bird, D., Yan, L., Vrotsos, K., Eliceiri, K., et al., “Metabolic mapping of MCF10A human breast cells via multiphoton fluorescence lifetime imaging of the coenzyme NADH” Cancer Res 65(19), 8766-73 (2005).

[16] Eliceiri, K., Fan, C., Lyons, G., White, J., “Analysis of histology specimens using lifetime multiphoton microscopy”. J Biomed Opt 8(3), 376-80 (2003).

[17] Adur, J., Pelegati, V., de Thomaz, A., Baratti, M., et al., “Optical biomarkers of serous and mucinous human ovarian tumor assessed with nonlinear optics microscopies” PLoS One 7(10), e47007 (2012).

[18] Pradhan, A., Pal, P., Durocher, G., Villeneuve, L., et al., “Steady state and time-resolved fluorescence properties of metastatic and non-metastatic malignant cells from different species” J Photochem Photobiol B 31(3), 101-12 (1995).