Quantitative Analyses on Second Harmonic Generation Microscopy Images of Collagen in Ex Vivo Basal Cell Carcinoma Samples in Comparison to Normal Skin

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Abstract: We carried out quantitative analyses including fast Fourier transform and CT-FIRE algorithms on images captured by second harmonic generation microscopy for the identification of basal cell carcinoma in *ex vivo* human skin samples.
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1. Introduction

Basal cell carcinoma (BCC) is the most frequent malignancy in Caucasians [1]. Although BCC has a very low metastatic rate, it can be highly destructive locally. Mohs micrographic surgery (MMS) is considered as the gold standard treatment of BCC. MMS utilizes repeated microscopic examinations of frozen sections of the tumor margins [2]. However, as MMS is a costly and time-consuming approach, there is a demand for efficient *in vivo* techniques for the assessment of BCC, which might be met in the near future by non-linear microscopy. Second harmonic generation (SHG) has been previously utilized for the imaging of BCC [3]. SHG signals are generated during the polarization of non-centrosymmetric molecules with high structural regularity as collagen of the skin [4]. In our present work, we compared various quantitative parameters and algorithms for the analysis of SHG images of collagen in *ex vivo* human BCC and healthy skin samples to evaluate their utility in the detection of BCC.

2. Materials and methods

BCC and healthy skin samples were collected from 10 patients by surgical excision and were kept in phosphate buffered saline. We captured SHG signals by a LSM 7 MP laser-scanning microscope (Carl Zeiss AG, Germany), with custom-modified detection optics. A Ti-sapphire laser (R&D Ultrafast Lasers Ltd, Hungary) was utilized, operating at 796 nm. A 405/20 nm band-pass emission filter was employed to separate SHG signal. A 20x water immersion objective (Carl Zeiss AG, Germany) was used. The imaging setup is described in more detail in Ref. [5].

We performed Fast Fourier Transformation (FFT) on the SHG images and converted the FFT output images into power plots. An ellipse was fitted to the power plots and collagen orientation index (COI) was calculated by COI = [1-(short axis/long axis)]. A COI value close to 0 reflects a healthy sample with isotropic behavior, while a value close to 1 indicates parallelly-oriented fibers. Collagen bundle packing (CBP) was expressed as CPB = 512*(1/*h*), where *h* is the distance between the centers of gravity of two first-order maxima of FFT plots [6]. IOD and FFT analyses were performed by ImageJ software (NIH, USA). Curvelet transform (CT) is a pre-processing mechanism, which de-noises the image while an automated fiber tracking algorithm (FIRE) extracts individual fibers [7, 8]. CT-FIRE v1.3 software (LOCI, USA) was run on the SHG images to extract the following parameters: fiber length, angle, width and straightness.

Statistical analysis was performed using GraphPad Prism v6.0 (GraphPad Software Inc., USA). The data were analyzed using Student's t-test after normal distribution was confirmed by F test. Results were considered significant if $p<0.05$.

3. Results

FFT images of BCC displayed significantly higher COI, indicating that the collagen fibers are less randomly arranged, while there was no difference in the CBP value (Fig. 1). CT-FIRE algorithm revealed increased collagen fiber length and decreased fiber angle in the BCC samples compared to controls (Fig. 2). The width and straightness of collagen fibers were similar in the BCC and the healthy skin samples.

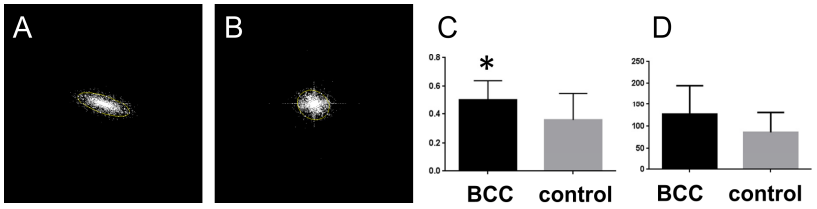

Fig. 1. A-B panels: Power plots of fast Fourier transformed second harmonic generation images. A: basal cell carcinoma (BCC), B: control skin; C: collagen orientation index; D: collagen bundle packing. Error bars represent standard deviation, *p<0.05.

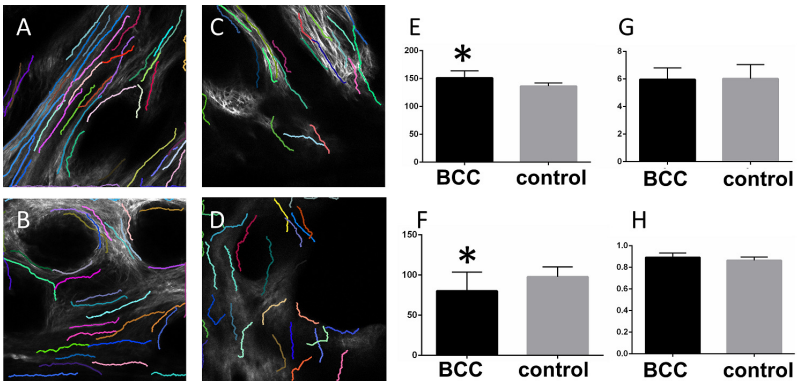

Fig. 2. Output images and data of CT FIRE algorithm performed on second harmonic generation images. A, B: basal cell carcinoma (BCC); C, D: control skin; E-H: results of CT-FIRE algorithm, parameters of collagen fibers: E: length, F: angle, G: width, H: straightness. Error bars represent standard deviation, *p<0.05. Size of the images is 300x300 μm^2.

4. Discussion

Recently, quantitative image analysis approaches have been utilized to evaluate the collagen structure in different conditions, including skin aging and different types of cancers [6-8]. However, these techniques have not been employed for the assessment of BCC. Therefore, in our present work, in order to investigate the applicability of these methods in BCC, we compared different collagen properties of *ex vivo* BCC samples to healthy skin. We found significantly higher COI and collagen fiber length and angle in the BCC samples utilizing FFT and CT-FIRE algorithms. These results are in line with previous findings which describe pronounced changes in the collagen structure of BCC [9]. Moreover, open source machine learning tools, such as R and Weka could be employed to improve these algorithms especially for the detection of BCC. In the future, these novel image analysis methods could be integrated in handheld nonlinear microscope systems [10], for sensitive and specific identification of BCC.

5. References

[1] G. Goldenberg, T. Karagiannis, J. B. Palmer, J. Lotya, C. O'Neill, R. Kisa, V. Herrera, and D. M. Siegel, "Incidence and prevalence of basal cell carcinoma (BCC) and locally advanced BCC (LABCC) in a large commercially insured population in the United States: A retrospective cohort study," J Am Acad Dermatol **75**, 957-966 e952 (2016).
[2] C. M. Clark, M. Furniss, and J. M. Mackay-Wiggan, "Basal cell carcinoma: an evidence-based treatment update," Am J Clin Dermatol **15**, 197-216 (2014).
[3] S. Seidenari, F. Arginelli, S. Bassoli, J. Cautela, A. M. Cesinaro, M. Guanti, D. Guardoli, C. Magnoni, M. Manfredini, G. Ponti, and K. Konig, "Diagnosis of BCC by multiphoton laser tomography," Skin Res Technol **19**, e297-304 (2013).
[4] M. Balu, C. B. Zachary, R. M. Harris, T. B. Krasieva, K. König, B. J. Tromberg, and K. M. Kelly, "In Vivo Multiphoton Microscopy of Basal Cell Carcinoma," JAMA Dermatol **151**, 1068-1074 (2015).
[5] D. Haluszka, K. Lorincz, N. Kiss, R. Szipocs, E. Kuroli, N. Gyongyosi, and N. M. Wikonkal, "Diet-induced obesity skin changes monitored by in vivo SHG and ex vivo CARS microscopy," Biomed Opt Express **7**, 4480-4489 (2016).
[6] S. Wu, H. Li, H. Yang, X. Zhang, Z. Li, and S. Xu, "Quantitative analysis on collagen morphology in aging skin based on multiphoton microscopy," Journal of biomedical optics **16**, 040502-040502-040503 (2011).
[7] J. S. Bredfeldt, Y. Liu, C. A. Pehlke, M. W. Conklin, J. M. Szulczewski, D. R. Inman, P. J. Keely, R. D. Nowak, T. R. Mackie, and K. W. Eliceiri, "Computational segmentation of collagen fibers from second-harmonic generation images of breast cancer," Journal of Biomedical Optics **19** (2014).
[8] Y. Liu, A. Keikhosravi, G. S. Mehta, C. R. Drifka, and K. W. Eliceiri, "Methods for Quantifying Fibrillar Collagen Alignment," in *Fibrosis* (Springer, 2017), pp. 429-451.
[9] M. A. Short, H. Lui, D. McLean, H. Zeng, A. Alajlan, and X. K. Chen, "Changes in nuclei and peritumoral collagen within nodular basal cell carcinomas via confocal micro-Raman spectroscopy," J Biomed Opt **11**, 34004 (2006).
[10] A. Krolopp, A. Csakanyi, D. Haluszka, D. Csati, L. Vass, A. Kolonics, N. Wikonkal, and R. Szipocs, "Handheld nonlinear microscope system comprising a 2 MHz repetition rate, mode-locked Yb-fiber laser for in vivo biomedical imaging," Biomed Opt Express **7**, 3531-3542 (2016).