Reviewer’s report

Title: Collagen reorganization at the tumor-stromal interface facilitates local invasion

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Reviewer: Thomas Dittmar

Reviewer’s report:

Comments Manuscript: Collagen reorganization at the tumor-stromal interface facilitates local invasion Provenzano et al.

In their manuscript the authors describe novel findings about tumor mediated reorganization of the collagen structure in mammalian tissue. To visualize collagen structures they performed multiphoton laser-scanning microscopy, whereby endogenous fluorophores were detected by multiphoton excitation and stromal collagen by second harmonic generation, respectively. By applying this techniques the authors were able to define three tumor-associated collagen signatures (TACS), that not yet have been detected with classical histological techniques, and which may provide novel hallmarks to locate and characterize tumors. Furthermore, the authors suggest that these TACS may serve as a part of a strategy to help identify and characterize breast tumors in animal and human tissues.

1) Is the question posed by the authors new and well defined? Yes!

2) Are the methods appropriate and well described, and are sufficient details provided to replicate the work? To study collagen distribution and structure within mammalian tissue (with or without breast tumors) the authors performed multiphoton laser-scanning microscopy (MPLSM), whereby endogenous fluorophores were detected by multiphoton excitation (MPE) and stromal collagen by second harmonic generation (SHG), respectively. Images were taken from fixed samples as well as from living animals. The advantage of MPLSM are sharp and clear images due to a reduced background of unspecific signals. The method is appropriate and well described. Sufficient details are provided to replicate this work.

3) Are the data sound and well controlled? The authors investigated an interesting topic. Tumor cell migration is a crucial process in metastasis formation and tumor cell mediated reorganization of the collagen structure might play a role in the initiation of this process. Unfortunately, the presented data are sometimes less informative (see below).

Figure 1-3: really excellent data/ images. However, bars (Fig. 1C-G and Fig. 2Ae) should appear in black instead of green (better contrast). The bar is missing in Fig. 1A, Fig. 2Ba, Bb, Bc, Bd, Be, and Fig. 3A-F, H. Can the authors explain why some data are presented as greyscale images whereas other data are shown in color? Figure 4-7: the data presented in these images are solely partially of good quality. The major point of criticism is that due to SHG imaging only reference images (as described by Hegerfeldt et al. (Ref. 21 and 51 (it’s a duplicate)) are shown, thus making it extremely difficult to detect tumor tissue and even single tumor cells within the collagen structure. This fact particularly applies to the figures showing higher magnifications (Fig. 5-6).

It is clear that the authors' aim is to focus on the particular collagen structure in dependence of tumor growth. However, if they want to show changes of the extracellular matrix caused by tumor cells/ tumor tissue they must provide data that tumor cells are localized in close proximity to collagen fibers or have already evaded into the stromal tissue, respectively. SND reflection images are excellent to resolve the collagen structure, but is solely partially suitable to visualize single tumor cells or the tumor mass. Thus, in particular for these data, which are the main topic of the work, the tumor mass and single tumor cells must be visualized (e.g. F-actin staining by Phalloidin as described by Hegerfeldt et al. (Ref. 21/ 51) or beta-1 integrin expression, which is an excellent target to visualize tumor cell-collagen interactions or by detecting NADP(H) as shown in supplementary Figure 2C) or the in vivo data must be confirmed with appropriate in vitro data as e.g. is shown in supplementary Figure 3. Here H&E data are confirmed with MPLSM data (or vice versa).

Figure 4: The tumor-stromal boundary is missing in Fig. 4Bb and Fig. 4Cb, Cc.

Figure 5: The labels â€œAâ€œ , â€œBâ€œ , and â€œCâ€œ should appear in â€œwhiteâ€œ instead of
The authors should use a different color to visualize e.g. tumor cells and the tumor-stromal boundary. The red color is hardly visible, which is also true for cyan (â€œâ€œ for tumor) and pink (â€œâ€œ for stromal). The authors should use a different color with a higher contrast to black and white, e.g. yellow as in Fig. 4.

Especially higher magnification images (Fig. 5E, 5F) should be confirmed by appropriate in-vitro data. Are 5E and 5F higher magnifications of 5D? If so, the particular areas should be marked by a rectangle.

Single collagen fibers are clearly visible but if they are really in contact with tumor cells (especially those cells that have been evaded into stromal tissue as shown in Fig. 5D (marked by â€œâ€œ)) is extremely difficult to see. This applies as well to these tumor cells that already have been evaded into stromal tissue (marked by â€œâ€œ). Dark spots (5D) or white spots (5E) are marked and it can not be deduced from the images that these structures are tumor cells. Thus visualization of tumor cells is necessary.

All scale bars are missing in Fig. 5!

Figure 6: The same criticism as for Fig. 5. A discrimination between stromal tissue and tumor tissue, especially evaded tumor cells (marked by a black arrowhead) is pretty difficult. Tumor cells must be visualized. The tumor-stromal boundary should be marked. B,C, and D show higher magnifications of A. The particular areas should be marked by a rectangle in â€œFig. 6Aâ€œ. What is shown in Fig. 6D? Scale bars are missing!

Figure 7: In Fig. 7C and 7D the interactions between tumor cells and collagen fibers should be analyzed by staining the beta-1 integrin expression of the tumor cells.

4) Does the manuscript adhere to the relevant standards for reporting and data deposition?
Yes!

Minor concerns:
Page 8, line 9, 10, 11, 13: “Fig. 4” must be “Fig. 2”
Page 11, line 4-7: “Furthermore, …” Which group of cells do the authors mean? The appropriate cells should be marked in Fig. 4.
Page 12, line 2-3: “Additionally, …” As stated out above, invading cells in direct contact with fibers are difficult to see. The authors indicate that some matrix disorganization was present but did not show appropriate results.

5) Are the discussion and conclusions well balanced and adequately supported by the data?
The discussion is well written and well balanced. Of course, the finding of different collagen structures might be helpful but I do not agree with the authors that TACS can be used as a standard hallmark for locating small tumors. MPLSM requires a certain equipment and due to the technical limitations the search for small tumors seems like the â€œlook for a needle in a haystackâ€œ. Moreover, the utilization of this technique in living animals is interesting but not is feasible in humans, except human tumor biopsies, which can be analyzed by this technique.

For me, this technique is more suitable to define the appropriate tumor grade and is therefore a helpful method to improve tumor classification and cancer treatment. As shown in supplementary Fig. 3. MPLSM data confirm, improve and support H&E data. Thus, TACS might be used a standard hallmark for the characterization of (small) tumors in addition to histological analyses. The conclusion should be revised. The authors should not go into detail, which mechanism might be responsible for matrix reorganization. Several pathways are known that induce and maintain cell migration such as e.g. EGF via EGFR/c-erbB-2 (HER2/neu) signaling, outside-inside integrin-signaling, etc. Additionally, these factors/mechanisms induce as well up-regulation and secretion of matrix metalloproteinases (MMPs), which are crucial for reorganization of the extracellular matrix.

6) Do the title and abstract convey what has been found?
Yes!

7) Is the writing acceptable?
Yes!

In summary, the authors present pretty interesting data. However, at its present form the manuscript is not suitable for publication in BMC Cancer. Major Compulsory Revisions are necessary.

What next?: Unable to decide on acceptance or rejection until the authors have responded to the major compulsory revisions

Level of interest: An article of importance in its field

Quality of written English: Acceptable
Statistical review: No

Declaration of competing interests:

I declare that I have no competing interests