Author's response to reviews

Title: Ultrasonic spectrum analysis for in vivo characterization of tumor microstructure changes in the evaluation of tumor response to chemotherapy using diagnostic ultrasound

Authors:

Chun-yi Lin (chunyilin58@hotmail.com)
Jian-wei Wang (jianweiwang85@hotmail.com)
Long-hui Cao (clhforever@hotmail.com)
Wei Zheng (weizheng_83@hotmail.com)
Yao Chen (chenyao-86@hotmail.com)
Zi-zheng Feng (Fengzz@sysucc.org.cn)
An-hua Li (anhuali@hotmail.com)
Jian-hua Zhou (zjh96421@hotmail.com)

Version: 2 Date: 19 March 2013

Author's response to reviews: see over
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Title: Ultrasonic spectrum analysis for in vivo characterization of tumor microstructure changes in the evaluation of tumor response to chemotherapy using diagnostic ultrasound

Authors:
Chun-yi Lin (chunyilin58@hotmail.com)
Jian-wei Wang (weizheng_83@hotmail.com)
Long-hui Cao (clhforever@hotmail.com)
Wei Zheng (weizheng_83@hotmail.com)
Yao Chen (chenyao-86@hotmail.com)
Zi-zhen Feng (fengzizhen06@yahoo.com.cn)
An-hua Li (anhuali@hotmail.com)
Jian-hua Zhou (*Corresponding author,zjh96421@hotmail.com)

Version: 2 Date: 18 March 2013

Author's response to reviews: see over
Reviewer's report

Title: Ultrasonic spectrum analysis for in vivo characterization of tumor microstructure changes in the evaluation of tumor response to chemotherapy using low-frequency ultrasound

Version: 1 Date: 25 January 2013

Reviewer: Rachel Sparks

Reviewer's report: 
Several grammatical errors: "Malignant tumors is one of the principal diseases", "the histology images of each 400 powers field were saved.
Corrections were made accordingly.

It would be useful for scatter plots or box and whisker plots of treatment groups with imaging and tumor characteristics to be shown to give a sense of the features. For instance it would be useful to show a scatter plot of tumor volume versus ultrasonic spectral parameters and spectral parameters versus histologic changes.
Scatter plots of spectral parameters versus cell nuclei density were made accordingly.

Additionally to make the claims about correlation between histologic changes and ultrasonic changes the authors should perform a regression analysis.
The correlation coefficient was computed accordingly.

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Needs some language corrections before being published

Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.

Declaration of competing interests:
I declare that I have no competing interest
Reviewer's report

Title: Ultrasonic spectrum analysis for in vivo characterization of tumor microstructure changes in the evaluation of tumor response to chemotherapy using low-frequency ultrasound

Version: 1 Date: 20 February 2013

Reviewer: Ronald Kumon

Reviewer's report:

I. Summary
This article uses ultrasound spectrum analysis to correlate changes in mid-band fit and slope with changes in tumor cell density which are induced as a result of chemotherapy in a murine breast cancer model. The authors conclude that conventional diagnostic ultrasound can be used to detect changes in tumor microstructure during chemotherapy.

II. Strengths and Weaknesses
The strengths of this article are:
1. It is an in vivo study.
2. It uses an ultrasound of frequency that is in the range of many existing diagnostic ultrasound systems.

The weaknesses of this article are:
1. Some similar work has been performed previously.
2. It does not provide a clear explanation of how the changes in the ultrasound spectrum parameters result from the changes in the tumor microstructure.
3. It uses a murine model, and so it remains to be determined if the tumors will respond to ultrasound similarly in humans in vivo.

III. Potential Clinical Importance
Improved monitoring of chemotherapy via quantitative ultrasound has the potential to provide more personalized treatment of cancer by allowing for the response of tumors to chemotherapeutic drugs to be more rapidly assessed.

IV. Overall Comments
The authors propose a well-defined question, use conventional and appropriate methods, and appear to have collected some sound data. The title and abstract largely convey the content of the manuscript. The background and/or discussion could be improved in regards to recent literature and the limitations of the work are
not clearly stated. The statistical methods would appear to be sufficient for the reported results. The writing is acceptable in most places. The study falls within the scope of BMC Cancer, and may be of interest to some of its readers.

IV. Specific Comments (see Revision lists)

V. Conclusion

This study reports some interesting results which are qualitatively consistent with some other similar studies. It appears to show a correspondence between changes in two spectral parameters and one tissue microstructural parameter, but these changes could be better correlated and quantified given their access to microstructural image data. As such, I would recommend that the authors undertake some further analysis prior to publication of this study, as specified, to potentially make their results more significant.

Major Compulsory Revisions

1. Background/Discussion: Several relevant references should be added to further place this study in the context of the relevant literature, and the authors should compare their results to these studies, as applicable:


   All of the above references were added in the revised article.

2. Methods/Results: What was the typical depth of the tumors? Were the spectral parameters corrected for attenuation? If the tumors were close to the surface, attenuation will have a minimal effect, but it should at least be stated if any
corrections were made. (If attenuation is an issue, perhaps the author can consider computing the spectral intercept, which is theoretically independent of attenuation or at least less sensitive.)

Due to the fact that the tumors were close to the skin surface and the center of the ROI was located about 0.2 cm below the skin surface, so whether attenuation compensation was made or not should not lead to a significant bias for a 6 MHz transducer. Therefore, no attenuation compensation was made in this study. This limitation was discussed in the revised article.

3. Methods, Paragraph 6: Can the authors provide an estimate of the error in ensuring that the plane of largest cross-section in the ultrasound image was the same as the plane of largest cross-section in the histological images? What was the out-of-plane resolution of the ultrasound probe? What was the thickness of the histology slices? This consideration is important because the entire study hinges on the fact that the same regions were sampled in the same places using both techniques.

The thickness of the histology slices was 5 μm and the out-of-plane resolution of the ultrasound probe was about 5 mm, so there were potential variations in matching ultrasound image planes with the histological slices. The reason that largest cross-section plane was used in both techniques is because we wanted to make ultrasound data more correspondent to histology measurement. This limitation was discussed in the revised article.

4. Methods, Paragraph 7: Can the author specify what criteria were used in Image Pro Plus to identify the nuclei (e.g., thresholds, geometric parameters, etc.)?

The criteria used in Image Pro Plus to identify the nuclei has been specify as follows: The hemotoxylin and eosin stained image segmentation was based on HSI parameters: Hue (0-255), Saturation (0-255), and Intensity (0-120). The segmented areas in the images were filtered to count blue nuclei. This filtering used thresholds as follows: area (minimum = 50 pixels) and box x/y (minimum = 0.5; maximum =2). The “split objects” function was used to separate cells touching each other.

5. Methods, Paragraph 7 & Results, Figure 3: In the Methods sections, the authors state that they calculated “the number of nucleus in each 400 powers field image.” However, in Results section and Figure 3, the authors report a “cell density” metric. Are the authors assuming that each cell only has one nucleus? If so, is that really a valid assumption in this particular tumor model? Please clarify.

The phrase “cell density” was changed to “cell nuclei density”.

6. Results, Figure 2: In the caption, the authors state that “No obvious difference was seen on the ultrasound images between the control (A) and the treated tumors (B).” However, to my eye the tumor in (B) looks to have a noticeably brighter grayscale intensity as compared to (A). This is borne out by the 15 to 20 dB increase in the midband fit. Can the authors compute the change in mean grayscale
intensity (or another image-based metric) to support their claim of “no obvious difference” in the ultrasound images?

Thanks for your suggestion. The gray scale intensity of the conventional B-mode ultrasound images was measured with the use of Adobe Photoshop, and Treatment with adriamycin (4 mg/kg once daily) significantly increased the gray scale intensity of the conventional B-mode ultrasound images as compared with control tumors ($P=0.009$).

7. Results, Figure 3: Can the authors show binary (black & white) images indicating which nuclei were identified by Image Pro Plus in both (B) and (C)?

A binary (black & white) image was provided to show the nuclei.

8. Results: Can the authors compute the effective scatterer size and concentration based on a reasonable model of scatterer geometry using the theory of Ref. 13? Given their access to the histological data corresponding to the ultrasound images, these estimates may allow the authors to determine more specifically what might be causing the changes in the spectral parameters. (As an example of such an correlative approach, see Lin K-W, Wang T-Y, Kumon RE, Deng CX, Xu Z, Hall TL, Fowlkes JB, Cain CA: Ultrasound backscatter spectral analysis provides image feedback for histotripsy tissue fractionation. In: 2011 IEEE Intl Ultrasonics Symp., pp. 33-36.) Also, can the authors compute other quantities from the histological images such as cell size, vacuole size, and nuclear size?

The limiting scatterer size should be three times smaller than typical axial resolution limits [1], so the limiting scatterer size for a 6-MHz linear transducer will be 85 $\mu$m which is much larger than the nuclear diameter (about 15 $\mu$m). Based on the reasonable model of scatterer geometry using the theory of Lizzi, the mean quantified scatterer diameter of the control and treated tumors in our study was 111.45±12.69 $\mu$m and 86.40±14.79 $\mu$m, respectively. Therefore, it was difficult to identify the histological texture of the scatterer for a 6-MHz linear transducer, and to specify the histological changes which caused the changes in the spectral parameters. Therefore, we do not think computing the effective scatterer size, concentration and the nuclear size would allow us to specify what should be causing the changes in the spectral parameters. This limitation was discussed in the revised article.


9. Results: Can the authors compute a quantitative correlation coefficient between the extent of the change in spectral parameters and the extent of the change in cell density the basis of individual cases? (For example, Lee et al. found that the correlation between the intercept and aggregate cell death was only moderate with $r = 0.42$.)

The correlation coefficient was computed accordingly.
Minor Essential Revisions
1. Methods, Paragraph 2, lines 5-6: Change awkward phrasing; perhaps better “…mice in the control group were given the vehicle control medium…”
   Change made as indicated by the reviewer.

2. Methods, Paragraph 7, lines 4-9: Change phrasing to minimize redundancy; perhaps better “The 400x histology images were then analyzed to measure the tumor cell density by counting the number of nuclei in each image using Image Pro Plus software (Image Pro Plus 6.0, Media Cybernetics, Silver Spring, MD, USA). The average count of ten 400x images was used for the statistical analysis.”
   Change made as indicated by the reviewer.

3. Methods, Paragraph 8, line 2: Change to “Kolmogorov-Smirnov test”.
   Done.

4. References: BMC Cancer style has the volume and issue numbers in bold.
   The references have been formatted in the BMC Cancer style.

Discretionary Revisions
1. Title and elsewhere: The use of the term “low frequency” is, unfortunately, somewhat relative. For example, 6 MHz may be low frequency for mouse studies, but would be considered high frequency of certain physiotherapeutic or HIFU studies. Perhaps it would be better to say “diagnostic ultrasound” or just “ultrasound” in the title, and specify the center frequency in the abstract.
   The term “low frequency” has been change to diagnostic ultrasound and the center frequency was specified in the abstract.

2. Methods, Paragraph 7: Change “x40” and “x400” to “40x” and “400x”, respectively.
   Done.

3. The reviewer thanks the authors for citing Ref. 12. The authors may wish to know that our group has a couple more recent publications on the same topic:
   Thanks. I have read these articles and learned a lot from them.
Minor Issues Not for Publication

1. Please insert comma before the conjunction between independent clauses (e.g., Background, Paragraph 1, line 14: “…tumor, and some…”) throughout the paper.
   Done.

2. Background, Paragraph 3, line 9: Change to “to differentiate”.
   Done.

3. Background, Paragraph 3, line 11: Correct mismatched parenthesis and bracket.
   Done.

4. Background, Paragraph 3, line 12: Change to “…with a high frequency transducer has…”.
   Done.

5. Methods, Paragraph 1, line 2: Capitalize “National Institutes of Health…”.
   Done.

6. Methods, Paragraph 1, lines 3-4: Capitalize “State Key Laboratory of Oncology in southern China.”
   Done.

7. Methods, Paragraph 1, line 8: Add hyphen in “phosphate-buffered…”
   Done.

8. Methods, Paragraph 3, line 3: Change bold to normal type “Centrifugated …”
   Done.

9. Methods, Paragraph 7, line 7: Change spelling to “Silver Spring”.
   Done.

10. Results, Paragraph 3, line 3: Add word to read “of the tumor cells…”
    Done.

11. Results, Paragraph 2: Change hyphens to minus sign in negative numbers (i.e., change “-10.66 dB/Mhz” to “#10.66 dB/MHz”).
    Done.

12. Discussion, Paragraph 1, line 16: Change to “…has the potential to detect earlier the tumors…”
    Done.

13. Discussion, Paragraph 3, line 22: Correct typographical error “dnsity” to “density”.
    Done.
14. Discussion, Paragraph 4, line 8: Change “breast cancer modes” to “breast cancer models”.
Done.

15. Discussion, Paragraph 4, line 11: Change verb from “changes after chemotherapy was” to “changes after chemotherapy were”
Done.

**Level of interest:** An article whose findings are important to those with closely related research interests

**Quality of written English:** Needs some language corrections before being published

**Statistical review:** Yes, and I have assessed the statistics in my report.

**Declaration of competing interests:**
I declare that I have no competing interests.