Author's response to reviews

Title: Relationship between morphological features and kinetic patterns of enhancement of the dynamic breast magnetic resonance imaging and clinico-pathological and biological factors in invasive breast cancer

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Version: 2 Date: 13 August 2009

Author's response to reviews: see over
August 10th, 2009

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Dear Sabina Alam,

Please find enclosed the re-revised version of our manuscript entitled “Relationship between morphological features and kinetic patterns of enhancement of the dynamic breast magnetic resonance imaging and clinico-pathological and biological factors in invasive breast cancer” (Reference: MS: 8418175082684879). We have revised the paper in response to all of the reviewer's minor and major comments, according to which it has been something modified. We are fully thanks their interesting comments on our manuscript.

Next, I would be pleased to answer to all the specific remarks suggested by the reviewers:

**Referee 1:**

**Reviewer:** Jeon-Hor Chen

**Reviewer's report:**
Minor Essential Revisions are needed for the following items. The author can be trusted to make these.

1. Some typos through the manuscript
   **Response:** All manuscript was revised by spelling.

2. Please define all MR imaging features using ACR BI-RADS MRI lexicon. This is the current standard to have the same communication language and to avoid confusion.
   **Response:** The lexicon used in the present study is very similar to ACR BI-RADS MRI (well-defined (regular); ill-defined (irregular or spiculated); Hom:
homogeneous; Het: heterogeneous). We did not use the ACR BI-RADS MRI lexicon because the majority of the lesions were previously diagnosed by biopsy of cytology. Nevertheless, we now reported the list of MR imaging features using ACR BI-RADS MRI lexicon in the revised version of the manuscript (In the Results section: page 13, paragraph 1).

3. In a long study period of 7 years (1999 - 2006), please clarify that all studied subjects had exactly the same MR sequences and parameters. Response: All studied subjects had exactly the same MR sequences and parameters, such as it is reported in the revised version of the manuscript following the Reviewer’s recommendation (In Materials and Methods section: page 5, last paragraph).

4. Please specify how many frames for the DCE-MRI. Response: We used the term of “sequence” instead “frame” in the original version of the manuscript. But following the Reviewer’s commentary, we use the term “frame” in the revised version, for avoiding confusion. A frame without contrast and six after contrast injection were performed. In addition, each frame had 64 slices. All of this information is now reported in the revised version of the manuscript (In Materials and Method section: page 6, paragraph 2).

5. For the placement of ROI, please further explain how you did it when the tumor showed rim enhancement. Response: The ROI was at least of three pixels and its placement was in the rim enhancement during the dynamic study when the tumors showed this finding (In the Materials and Methods section: page 7, paragraph 3).

6. Please clarify the HER-2 staining. Was it by IHC or by FISH? Response: The HER-2 staining was made by immunohistochemistry with rabbit polyclonal antibody from Dako and for the assessment we use the Herceptest scoring guidelines. According to that a tumor was reported 2+ when a weak to moderate complete membrane staining is observed in > 10% of tumor cells. These cases were classified as equivocal and required confirmation by FISH and a tumor was reported 3+ when a strong complete membrane staining is observed in > 10% of tumor cells. These cases were classified as positives and confirmation was not required. All of these data are now provided in the revised version of the manuscript (In the Material and Method section: page 11, paragraph 3).

7. Since one of the major findings of this study was the correlation of the kinetic enhancement and the MVD, details of how these two parameters were measured and compared in the exact tumor location are needed, especially for a tumor showing heterogeneous enhancement. Response: It is difficult to assess exactly the tumor localization evaluated by MRI and histologically. Nevertheless, we consider that we performed a reasonable correlation between both types of evaluations. The ROI was placed into the area that exhibits strongest enhancement on the first post-contrast image (In the Material and Method section: page 7, paragraph 3). On the other hand, with regard to micro-vessel density (MVD) evaluation, we examine five
fields per core, corresponding to areas without necrosis and of higher immunostaining with anti-CD34, with a final area of 1 mm². We obtain a total score and this is the value of MVD in each tumor. More details on this are provided in the revised version of the manuscript (In the Material and Method section: page 11, paragraph 2).

8. In your results, "we found that tumors showing a maximum enhancement peak at two minutes or longer, had a significant higher MVD count than those ones that reached this maximum point before two minutes (p=0.012)", although you have some explanation in the Discussion section, more detailed explanation of how your results were different from previous studies are needed.
Response: In accordance to Reviewer's suggestion, we reported more details of how our results are different from previous studies. Such as it is reported in the revised version of the manuscript “...it is important to consider that the divergent results may be a consequence of differences in the techniques of microvessel quantification as well as differences in the investigated tumor area. This latter aspect is important especially for a tumor showing heterogeneous enhancement. It is known that there may be discrepancies in MVD estimation when it is evaluated in the periphery or in the centre of the tumors. Thus, for example, it has been recently reported that the characteristic enhancement in the periphery of breast carcinomas at MRI is not caused by an elevated MVD in the tumor periphery but rather by a lower MVD in the tumor center [16]. We have analyzed the areas of higher MVD, by immunostaining with anti-CD34, in the tumoral center, and these were correlated with the area that exhibits strongest enhancement on the first post-contrast image. Therefore, we consider that our finding may be because the highest MVD delayed the display of the maximum enhancement capacity of the tumors" (In Discussion section: page 17 at the end of the single paragraph and page 18, paragraph 1).

9. Again, your results -- "The percentage of tumors with vascular invasion or with high mitotic index was significantly superior among those showing a low percentage of maximum enhancement (#150%) before two minutes than in those showing a high percentage (>150%) of enhancement rate at that time interval (p=0.016 and p=0.03, respectively", need to have some discussion why you have the findings.
Response: Such as it is now reported in the revised version of the manuscript: “These associations may be explained due a lower number of functional vessels because the occupation of these ones by cancerous cells with high proliferate rate, which prevent a fast passage of paramagnetic contrast” (In the Discussion section: page 19, paragraph 1).

10. The description - "These findings seem to indicate that MRI could provide prognostic information independent of those classical factors" is too strong and without data support in your study. Please modify.
Response: In accordance to the Reviewer recommendation, we modify this sentence, as follows: “These findings seem to indicate that MRI could provide complementary prognostic to those provided by the classical factors” (In the Discussion section: page 16, paragraph 3).
11. Please add a paragraph of study limitations in the end of the Discussion section.
Response: Following the Reviewer’s indication, we added a paragraph of study limitations in the end of the Discussion section: “Limitation of the present study is the difficult to assess exactly the tumor localization evaluated by MRI and histologically. Nevertheless, we consider that we performed a reasonable correlation between both types of evaluations. Certainly there are other possible parameters for evaluating MRI and both histological and biological aspects of breast carcinomas, which should be investigated in futures studies. In addition, prospective studies are necessary to assess the potential value of MRI parameters as prognostic factors in breast cancer”. (In the Discussion section: page 20, last paragraph).

12. The conclusion should be expanded regarding the major findings of this study, how they are adherent, or against, to the existing literature, and what are the potential results which may be helpful for patient management.
Response: Following the Reviewer recommendation, we performed a more complete conclusion at last of the Discussion section (page 21, first paragraph): “In conclusion, especially relevant are our findings that variations in the dynamic MRI parameters seem to be associated with parameters indicatives of tumor aggressiveness, such as high MVD count, vascular invasion, high mitotic index in breast cancer or peritumor inflammation. Therefore, our results are in accordance with previous report indicating the potential value of dynamic MRI for better characterizing breast cancer”.

Referee 2:

Reviewer: Gary Tse
Reviewer’s report:
This is an interesting study on the correlation of MRI with DCE and several important biological parameters of breast cancers. Some of the findings are quite novel, however there are several issues that the authors need to address. Major compulsory revision

Introduction. The authors stated that studies on DCE-MRI and tumor characteristics were few, and it is not true. There are many studies in the literature (e.g. Filippo Montemurro, 2007; Roka Matsubayashi, 2000; Mitsuhiro Tozaki 2004; etc.) The authors need to update the literature background search, and put forth how the current study differs from those in the literature.
Response: Following the Reviewer’s suggestion, we changed the second paragraph of the instruction adding the references recommended, as well as these of Tuncbilek et al, 2005 and of Lee et al., 2008. Likewise, we put forth how the current study differs from those in the literature: “…In addition, there are studies indicating that dynamic contrast-enhanced MRI help to predict prognostic factors and/or biological activity of breast cancer by revealing morphological features and enhancement parameters of the primary tumors.
Thus, MRI parameters has been associated with histological grade (Tuncbilec, 2005; Lee, 2008), angiogenesis, degree of fibrosis (Tuncbilec, 2005), negative expression of estrogen receptor of progesterone receptor (Lee, 2008), HER-2-overexpression (Montemurro, 2007) or expression of vascular endothelial growth factor (VEGF) (Matsubayashi, 2000). In this context, the objectives of this study were to investigate the relationship between the MRI features of breast cancer and some other of its clinicopathological and biological characteristics, such as vascular invasion, peritumoral inflammation or VEGF-receptor-1 and 2 (In the Introduction section: page 3, last paragraph; and page 4, first paragraph).

Materials and Methods.

a. Early breast cancer cases were selected. How were they selected, and what were the criteria for defining a case as early.
Response: Following the Reviewer’s suggestion, we provide one more complete information in the revised version of the manuscript: “This study comprised 68 women consecutively diagnosed of early invasive breast cancer (without distant metastasis at time of initial diagnoses) and treated between 1999 and 2006. Initially, the lesions were detected by physical examination, mammography, or ultrasonography. All of the women did not receive any type of neoadjuvant therapy.” (In Materials and Methods section: page 5, paragraph 1).

b. Did the authors classify the tumors into different histotypes? Breast cancer is a heterogeneous disease, and not all tumors behave like the NOS type.
Response: All tumors included in the present study were invasive breast carcinomas. We listed all of the different histological types in the Materials and Methods section (page 5, paragraph 1). In addition, we mentioned in the Results section that significant differences in MRI parameters were not found between the different histotypes, as data not shown (page 13, paragraph 2).

c. The authors have not defined how to assess the micro-vessel density (MVD)
Response: we examine five fields per core, corresponding to areas without necrosis and of higher immunostaining with anti-CD34, with a final area of 1 mm². We obtain a total score and this is the value of MVD in each tumor. More details on this are provided in the revised version of the manuscript (In the Material and Method section: page 11, paragraph 2).

d. HER2 assessment – most people use 30% cutoff as positive rather than 10%.
Response: The HER-2 staining was made by immunohistochemistry with rabbit polyclonal antibody from Dako and for the assessment we use the Herceptest scoring guidelines. According to that a tumor was reported 2+ when a weak to moderate complete membrane staining is observed in >10% of tumor cells. These cases were classified as equivocal and required confirmation by FISH and a tumor was reported 3+ when a strong complete membrane staining is
observed in > 10% of tumor cells. These cases were classified as positives and confirmation was not required. All of these data are now provided in the revised version of the manuscript (In the Material and Method section: page 11, paragraph 3).

**e. How was the histologic grade assessed? Modified Bloom and Richardson? Did the authors also assess the nuclear grade? This has to be listed in the M&M section.**

**Response:** The histologic grade was assessed according to criteria reported by the Nottingham modification of Bloom and Richardson score (SBR). Reference: Dixon AR, Ellis IO, Elston CW, et al. A comparison of the clinical metastatic patterns of invasive lobular and ductal carcinomas of the breast. *Br J Cancer.* 1991; 63:634-635. (In the Material and Method section of the revised version of the manuscript: page 5, paragraph 1). On the other hand, we mentioned in the Results section that significant differences in MRI parameters were not found with regard to nuclear grade, as data not shown (page 13, paragraph 2).

**Results.**

**a. Some authors reported correlation of grade to enhancement pattern.**

**The authors need to comment the reason(s) for this disparity (Tuncbilek N, 2005).**

**Response:** As can be seen in Table 1, we also found higher kinetic features in grade I tumors than in grade III tumors, such as peak of maximum enhancement <2’ of type III curve. However, the differences in these parameters did not achieve significant differences. We consider that this may be due to the small size of sample for this comparison as well as to the known inter-observer variation in histological grade evaluation (1,2). These commentaries are now reported in the revised version of the manuscript (In the Discussion section: page 16, last paragraph; and page 17, first paragraph).


**b. The text on the possible reason for increased MVD and delayed maximal enhancement was difficult to understand, suggest further explanation (second half of p.15)**

**Response:** Following the Reviewer’s recommendation, we added more explanation on this in the revised version of the manuscript: “This may be because the paramagnetic contrast spends more time in to fill in the tumors very vascularized (In the Discussion section: page 18, paragraph 1).

**c. VEGF has a prominent and important role in enhancing vascularity permeability, thus contrast diffusion. This fact may warrant some discussion.**
Response: Following the Reviewer's suggestion we added a sentence of this in the revised version of the manuscript (In the Discussion section: page 18, paragraph 1).

d. Was there any correlation between nuclear grade with the DCE-MRI parameters? Was there any correlation between VEGF and MVD?
Response: Our results did not show significant association between nuclear grade and the DCE-MRI parameters, such as is now reported in the revised version of the manuscript (In the Results section: page 14, paragraph 2). On the other hand, we found not significant correlation between MVD and VEGFR-1 or -2.

Minor essential revisions
Materials and Methods.
a. It is interesting to note that axillary dissection was done in all cases as these were defined as early breast cancer. Was sentinel node assessment performed in the institution?
Response: Thank you!, for the Reviewer observation. In our institution we begin to make the selective biopsy of sentinel node over 2006. Therefore, axillary Dissection did not make in all cases, such as it was showed for error in the revised version of the manuscript. Only the criteria of then histopathologically assessed axillary lymph nodes was considered for these cases with axillary deissection. Nevertheless, for to avoid confusion, this criteria was deleted in the revised version of the manuscript (Page 5, paragraph 1).

b. For VEGFR assessment, 400x power objective? Please clarify
Response: We use 400x power objective in two fields per core (In the Materials and Methods section of the revised version of the manuscript: page 10, paragraph 2).

c. For VEGFR assessment, the authors tried to average out the staining score. Giving the known tumor heterogeneity of breast cancer, it is not better to concentrate on the highest staining area?
Response: Fields were selected by searching for the highest staining area and finally we average the staining score (In the Material and Method section of the revised version of the manuscript: page 10, paragraph 2).

Results.
a The authors need to include in the study cohort whether or not there was DCIS component.
Response: DCIS component was present in 16 cases (23.5%). However our results did not show significant associations between this associated component and MRI parameters, such as it is now reported in the revised version of the manuscript (In the Material and Method section: page 5, paragraph 1; and in the Results section: page 14, last paragraph, as data not shown).
b. Some authors reported correlation of grade to enhancement pattern. The authors need to comment the reason(s) for this disparity (Tuncbilek N, 2005)  
Response: As can be seen in Table 1, we also found higher kinetic features in grade I tumors than in grade III tumors, such as peak of maximum enhancement <2′ of type III curve. However, the differences in these parameters did not achieve significant differences. We consider that this may be due to the small size of sample for this comparison as well as to the known inter-observer variation in histological grade evaluation (1,2). These commentaries are now reported in the revised version of the manuscript (In the Discussion section: page 16, at the end of last paragraph; and page 17, paragraph 1).


c. Was there any correlation between nuclear grade with the DCE-MRI parameters? Was there any correlation between VEGF and MVD?  
Response: Our results did not show significant association between nuclear grade and the DCE-MRI parameters, such as is now reported in the revised version of the manuscript (In the Results section: page 14, paragraph 2). On the other hand, we found not significant correlation between MVD and VEGFR-1 or -2.

Referee 3:

Reviewer: Sally Lee
Reviewer’s report:
Minor Essential Revisions

1. In the Abstract section:
(a) Please specify the range of smaller tumor size.  
Response: We specify the range in the revised version of the manuscript: T1: <2cm) (In Abstract section: page 2, paragraph 2).

(b) Tumors showing a maximum enhancement peak at two minutes – please specify that it’s two minutes after injecting an agent.  
Response: …after injecting the contrast (In Abstract section: page 2, paragraph 2).

(c) The <= sign did not appear in the pdf file.  
Response: this sign was corrected (In Abstract section: page 2, paragraph 2).
2. In the Materials and Methods section:
(a) Not sure what it meant by “68 consecutive women”.
Response: ...Were women consecutively diagnosed of breast cancer (In Materials and Methods section: page 5, paragraph 1).

(a) Please specify what are the other varieties of tumours that have been identified.
Response: these other were two mucinous, one medullar, one tubullar and one papillar (In Materials and Methods section: page 5, paragraph 1).

(b) Spelling for "General Electric Medical Systems".
Response: The term was correctly written (In Materials and Methods section: page 7, paragraph 1).

(c) Spelling for “Sagittal”.
Response: The term was correctly written (In Materials and Methods section: page 6, paragraph 1).

(d) The unit for TR and TE?

(e) Please re-arrange the sentences for describing the MR imaging:
Acquisition of dynamic imaging started 10s after contrast injection, following by six sequences, which lasted 80 seconds each. Was there a gap between each sequence? If so, how long was it?
Response: Following the Reviewer’s recommendation, we re-arrange the sentences for describing the MR imaging: “Before administration of contrast material, T1-weighted sequences were acquired in the axial plane (FSPGR -fast spoiled gradient echo- 3D; FA -flip angle-, 10°; TR, 9.9 ms; TE, 4.2 ms; NEX, 1; 2-3 mm slice thickness with no gap; 512 x 192 matrix; in-plane resolution, 0.6 x 1.8; frequency was in the anteroposterior direction). Acquisition of dynamic imaging started 10s after the intravenous injection of 0.2 mmol per kilogram body weight of gadopentetate dimeglumine (Gd-DTPA) (Magnevist; Schering, Madrid, Spain), followed by a 20 ml saline solution flush, at an injection rate of 2 mL/s, following by six series, with lasted 80s each for a total imaging time of slightly over nine minutes. The injection unit contained no magnetic components and operated with pressurized air.” (In Materials and Methods section: page 6, paragraph 2).

Image Analysis:
(g) Please specify the range, in terms of pixels, for the region of interest.
Response: The ROI size was always greater than three pixels, and without upper limit (In Materials and Methods section: page 7, paragraph 2).

(h) The usage of superscript should be applied to the formula of rSlc.
Please include the citation for the enhancement formula as well as the signal intensity curve categories.
**Response:** Following the Reviewer recommendation, we usage superscript. In addition, we included the citation of Kuhl et al. Radiology 1999; 211: 101-110 (In Materials and Methods section: page 7, paragraph 3).

(i) Duration consideration. What was the reason for looking into every 2 minutes?
(citation from the literature)
**Response:** …within 2 to 4 minutes (including 2 and 4 minutes), within 4 to 6 minutes (>|4 minutes and 6 minutes inclusive), and after 6 minutes;…(In Materials and Methods section: page 8, paragraph 1). This was a formule adopted by us.

Tissue arrays and immunohistochemistry
(j) In the second paragraph, what does “H&E” stand for?
**Response:** We used the whole term in the revised version of the manuscript (“Haematoxylyn and eosin”) (In Materials and Methods section: page 9, paragraph 2).

Data analysis and statistical methods
(k) Please give details on how patients were subdivided into groups based on different clinical and pathological parameters.
**Response:** Following the Reviewer recommendation, we give these details (In Materials and Methods section: page 12, paragraph 1).

Results
(a) Please specify what are T1 and T2 tumors in the first paragraph.
**Response:** …T1 (<2 cm in size) tumors (28.6%), whereas none of the ≥T2 tumors (≥2 cm)... (In the Results section: page 13, paragraph 1).

(b) Third paragraph: spelling of minute.
**Response:** The spelling was corrected (In the Results section: page 14, paragraph 2).

Discussions
(a) Please specify what T1 and T2 stand for in the second paragraph as well as in Table 1.
**Response:** … tumors smaller than 2 cm (T1), whereas none of the T2 tumors greater than 2 cm (≥T2) showed (In the Discussion section: page 16, paragraph 2).

(b) "MVD reflects the angiogenesis activity which constitutes a prerequisite for the growth of malignant tumors beyond a certain size" -> What was the certain size?
Response: …greater than 2 millimetres… (In the Discussion section: page 17, paragraph 2).

Finally, we would like to acknowledge the opinions of the experts who reviewed our manuscript.

Yours sincerely,

Dr. Francisco J. Vizoso