Author's response to reviews

Title: Expression of centromere protein F (CENP-F) associated with higher FDG uptake on PET/CT, detected by cDNA microarray, predicts high-risk patients with primary breast cancer

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Version: 5 Date: 20 October 2008

Author's response to reviews: see over
Dear Dr. Melissa Norton,

Manuscript ID : 7216131472125411

Title: Expression of centromere protein F (CENP-F) associated with higher FDG uptake on PET/CT, detected by cDNA microarray, predicts high-risk patients with primary breast cancer

Thank you very much for your E-mail dated 3 September, 2008. We have improved the contents of our manuscript according to the referees' comments as follows. Because Takayuki Kobayashi, M.D. has critically reviewed the manuscripts and improved the manuscript, we would like to add him as one of the co-authors.

# 1. Reviewer's report

Title: Expression of centromere protein F (CENP-F) associated with higher FDG uptake on PET/CT, detected by cDNA microarray, predicts high-risk patients with primary breast cancer

Version: 3 Date: 3 September 2008
Reviewer: Donal Brennan

Reviewer’s report:

The authors have presented a study whereby they evaluated used PET imaging to identify tumours with a high SUV which they believe to be a good prognostic tool. They followed on to identify a number of genes associated with a high SUV and then validate these using rtPCR and IHC

Major Compulsory Revisions

1. There a number of misrepresentations in the introduction mainly around the phrase "primary systemic chemotherapy" and the use of the Mammaprint and Oncotype DX assays. For example the authors say these are "sufficiently established" which is wrong. In general the introduction is poorly written and needs to be completely revised.

   We have extensively revised descriptions in introduction as follows: In the sentence of the introduction in line 8, page 4, we have corrected ‘primary systemic chemotherapy (PSC)’ to ‘primary systemic therapy (PST)’. In line 14, page 4, we have corrected ‘sufficiently established methods of’ to ‘going to be used widely for’. In line 19, page 4, we have corrected ‘a widely used imaging device with a noninvasive approach’ to ‘a noninvasive imaging device widely used’. In line 19, page 4, we have corrected ‘FDG uptake on PET is a highly reproducible and quantitative parameter of tumor glucose...’
metabolism, which employs the standard uptake value (SUV) to ‘FDG uptake on PET, quantitated by the standardized uptake value (SUV), is a highly reproducible parameter of tumor glucose metabolism’. In line 12, page 5, we have corrected ‘In this study,’ to ‘The present study’. In line 19, page 5, we have corrected ‘select’ to ‘identify’.

The description of Mammaprint™ and Oncotype DX® was mistaken. We have corrected Oncotype DX™ in line 13, page 4 and Mammaprint® in line 15, page 4.

2. As there are three cohorts used in the it is hard to follow. They need to be outlined in detail in the materials and methods section and a table needs to be provided to allow reviewers to examine them for differences between the cohorts

In the materials and methods, we have added the sentences that “Clinicopathological features of patients classified according to SUV are shown in Table 1. Regarding to age, pT-factor, pN-factor, nuclear grade, ER, and HER2, no statistical difference were detected between 2 groups.” in page 8, lines 5-7. In addition, the novel Table 1 entitled ‘Clinicopathological features of 38 patients with primary breast cancer subjected to cDNA microarray and RT-PCR analyses’ has been added in this paper.

Previous Table 1, Table 2, and Table 3 have corrected to Table 2, Table 3, and Table 4, respectively.
3. My major issue regarding this paper is regarding the cDNA microarrays. The bioinformatic approach outlined in the paper is fundamentally flawed and needs to be addressed. Initially the groups are made up of different numbers of tumours 14 versus 24. No clinical data is given regarding the nuclear grade, tumour size or nodal status in the two groups.

We have added in lines 16-21, page 6 that ‘Initially, a total of 48 samples comprising 24 high SUV tumors and other 24 low SUV tumors, that were matched with regard to pT and pN factors, were subjected to RNA isolation. A sufficient volume of total RNA were extracted from all 24 samples of high SUV tumors and 14 (58%) of 24 samples of low SUV tumors. Therefore, a total of 38 samples were used for the cDNA microarray study.’

As written above, clinicopathological data regarding nuclear grade, tumor size, or nodal status in the 14 tumors of the low SUV group and in the 24 tumors of the high SUV group have been given in the newly presented table 1.

There is no information about how the tumours were stored following surgery - were they snap frozen or stored in RNA later?

We have added the sentences of the materials and methods that ‘the tissue samples obtained from surgical specimens were immediately frozen in liquid nitrogen until RNA isolation.’ in line 9-10, page 8.
If the SUV high group has a larger number of high grade T2 tumours obviously cell cycle related genes such as CENPF.

In the analysis of cDNA microarray, T-factor and nuclear grade were not statistically different between the high SUV group and the low SUV group as presented in novel Table 1.

Additionally no information is given about the approach used to identify the top 20 genes -were the data normalised, why was a cut off of 1.7 fold used?

We defined genes with Cy3 : Cy5 ratios of 3.0 or greater in signal intensity as up-regulated genes because of the following reasons:

We first identified 20 genes, that showed the Cy3 : Cy5 ratios of 1.7 or greater, as the candidate genes that were upregulated in the high SUV tumors (Table 2). In the two genes that showed the Cy3:Cy5 ratios of 3.0 or greater, i.e., CENP-F and CDC6, we could validate their upregulation in the high SUV tumors by RT-PCR as mentioned below.

On the other hand, in other candidate genes, e.g., gtf2b (Cy3:Cy5 ratio 2.79), KRT5 (Cy3:Cy5 ratio 2.05), MMP9 (Cy3:Cy5 ratio 1.95), and PLAU (Cy3:Cy5 ratio 1.78), their up-regulation could not be validated by means of RT-PCR. Furthermore, a housekeeping gene GAPDH was also ranked in the candidate genes with a Cy3:Cy5 ratio of 2.18 by cDNA microarray analysis. Therefore, we chose the cut-off value of 3.0.
We have added these sentences in lines 7-19, page 9 in the revised manuscript.

4. The TMAs used in this study were constructed using a single 2mm core from tumour blocks. It is debatable how well this approach accounts for tumour heterogeneity given that current recommendations are to take a minimum of 3 1mm cores.

Thank you for your suggestion. The description of the sentence in line 5, page 10 that ‘a single tissue core with a diameter of 2.0 cm was punched out’ was mistaken.

Correctly, the TMA was constructed using two 2 mm-cores from different two areas in each of 253 surgical specimens. We have corrected the sentences that ‘two tissue cores with a diameter of 2.0 mm were punched out’ in line 7, page 11. We believe the TMA used in the present study met the requirement of current recommendation.

I can't see the benefit of this paper in predicting the need for or response to adjuvant chemotherapy. My own opinion is that CENPF is probably a marker of high grade aggressive tumours, however I don't understand what SUV adds to it as these patients will all have surgery prior to any chemotherapy. My own feeling is that PET imaging may help to identify that small number of patients who will show a complete pathological response following neoadjuvant therapy.
As you pointed out, CENPF is probably a marker of high grade aggressive tumours. By molecular approach of this study, we confirmed that CENPF as a cell-cycle biomarker was one of important factors relevant to high SUV levels of tumors.

We agreed with the reviewer’s opinion that PET imaging may help to identify the patients who will show a complete pathological response following neoadjuvant therapy. We have added the following sentence in the last of discussion in lines 15-17, page 20: “FDG PET imaging may be helpful to predict or identify the patients who will show a pathological complete response following neoadjuvant therapy.”

Level of interest: An article of insufficient interest to warrant publication in a scientific/medical journal

Quality of written English: Not suitable for publication unless extensively edited

The present manuscript has been edited by an English native.

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

Declaration of competing interests:

I declare that I have no competing interests
Reviewer's report

Title: Expression of centromere protein F (CENP-F) associated with higher FDG uptake on PET/CT, detected by cDNA microarray, predicts high-risk patients with primary breast cancer

Version: 3 Date: 9 September 2008

Reviewer: Emmanuelle Jauffret

Reviewer's report:

Ueda et al reported the identification and study of 2 molecules differentially expressed between groups of breast cancers that respectively display high and low standardized uptake value (SUV) using FDG PET/CT. They validated using RT PCR the different mRNA level of CENP-F and CDC6 between both groups and by immunohistochemistry the overexpression of CENP-F in high SUV group of patients.

High CENP-F level in IHC is a bad prognostic marker in DFS and OS, and is CENP-F level is an independant prognostic factor of recurrence.

The data are interesting, but minor points need to be answered prior to publication:

1) as SUV is correlated to glucose metabolism, isn't it a problem to use N0 and N+ patients in the study? is the level of SUV the same in N0 and N+ patients?

In the analysis of cDNA microarray of the current study, pT and pN factors were matched between high SUV groups and low SUV group. There were statistical differences between the high SUV tumors and the low SUV tumors with regard to the N factor as shown in the novel Table 1.

2) are we sure that patient did not receive any chemotherapy before surgery? this
point needs to be precised

We have added the sentence in lines 10-11, page 6 that ‘These patients did not receive any systemic therapy before surgery’

3) does core biopsy modify the level of SUV? in that case, it as to be taken into account to compare level of SUV

We have added in the sentence that ‘FDG PET/CT examination was performed at an interval of 2 weeks or more after a core needle biopsy.’ in line 10, page 6. We consider that the CNB performed to the patients did not effect on the FDG PET/CT results because: 1. CNB was performed to all patients, and 2. FDG PET/CT examination was performed at an interval of 2 weeks or more after a core needle biopsy.

4) What about the treatment conducted in the TMA population

this population is used to calulate survival data and as CENP-F is in relation with cell cycle, it is more relevant to compare patients who had or not chemotherapy together

We couldn’t investigate in detail about the correlation of CENP-F with patients who had or not chemotherapy in this TMA population by the following reasons.

From 1990 to 1995, in Japan, we had no standard adjuvant chemotherapy including CMF or anthracycline-based regimens to a majority of patients. In principle, node-negative cases did not receive adjuvant chemotherapies. Some patients having node-positive breast cancer had received oral 5-FU chemotherapy, but the dose and duration varied from case to case. Therefore, we could not analyze the correlation of CENPF with the effect of standard adjuvant chemotherapy.

5) for the cDNA arrays: does the authors know if the hierarchial clustering of samples separate high and low SUV?

We didn’t plan to construct a hierarchical clustering of samples to separate high and low SUV tumors in the present study.

6) is there a correlation with molecular subtypes (Basal-like, luminal-like...) and
In our study, triple-negative tumors (including basal-like and normal breast subtypes) and HER2-overexpressed tumors showed higher SUV levels compared with luminal-A tumors. On the other hand, luminal-A tumors had a wide range of SUV levels. The legend for supplementary figure has been additionally presented in the manuscript in lines 1-8, page 26.

In the genes overexpressed in high SUV, we can found "basal" genes (KRT5, ACTA1, COL7A1). is it possible to calculate this correlation?

There was no correlation between high SUV and KRT5 expression by RT-PCR. We did not perform RT-PCR assays for ACTA1 and COL7A1.

what about the genes underexpressed? RE-related genes?

We have identified 8 sequences that showed 0.33 or less of Cy5 : Cy3 ratios in the tumors as listed below. There were no ER-related genes in the list.

<table>
<thead>
<tr>
<th>ratio</th>
<th>Gene_name</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.15</td>
<td>galactosidase, alpha (GLA)</td>
</tr>
<tr>
<td>0.23</td>
<td>cartilage intermediate layer protein, nucleotide pyrophosphohydrolase (CILP)</td>
</tr>
<tr>
<td>0.24</td>
<td>secreted frizzled-related protein 4 (SFRP4)</td>
</tr>
<tr>
<td>0.25</td>
<td>hypothetical protein FLJ22418 (FLJ22418)</td>
</tr>
<tr>
<td>0.28</td>
<td>ephrin-A1 (EFNA1)</td>
</tr>
<tr>
<td>0.30</td>
<td>-</td>
</tr>
<tr>
<td>0.33</td>
<td>amphiregulin (schwannoma-derived growth factor) (AREG)</td>
</tr>
<tr>
<td>0.33</td>
<td>hypothetical protein FLJ21174 (FLJ21174)</td>
</tr>
</tbody>
</table>

in that case, is CENP-F expression independant of molecular subtype?

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

There was no significant correlation between CENP-F expression and molecular subtypes (luminal, HER2-overexpressed, and basal-like tumors) ($p = 0.5$).

Quality of written English: Acceptable
Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:

'I declare that I have no competing interests'