Author's response to reviews

Title: Establishment of Hewga-clear cell sarcoma, a new clear cell sarcoma cell line, and investigation of the antitumor effects of pazopanib on Hewga-CCS: an in vitro and in vivo study

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Author's response to reviews: see over
June 3, 2014  
Editorial team  
*BMC Cancer* 

Dear Editor,  

With this letter, we are respectfully resubmitting our manuscript No.: 1048507155121042 entitled “Establishment of a novel clear cell sarcoma cell line (Hewga-CCS), and investigation of the antitumor effects of pazopanib on Hewga-CCS” by H. Outani et al. for your consideration for the publication as an original research paper in *BMC cancer*.  

In accordance with numbers of comments from five reviewers, we carried out several new experiments, made new figures and rewrote the revised manuscript to address their comments. Text changes are marked by red. We also attempted to answer each query one by one from the reviewers’ points summarized in the attached excel file. We also sent the Supplemental Figure for reviewer 1, 2, and 3 only for answering to the inquiry from reviewer 1, 2, and 3 to editorial@biomedcentral.com. Please notice that these files will not be for publication.  

This manuscript has not been published elsewhere and is not under consideration by another journal. All the authors have approved the final version of the manuscript, agree with its submission to *BMC Cancer*, and report no conflicts of interest.  

We believe that the findings of this study are relevant to the scope of *BMC Cancer* and will be of interest to its readership. The manuscript has been carefully reviewed by an experienced editor whose first language is English and who specializes in editing papers written by scientists whose native language is not English.  

We look forward to hearing from you at your earliest convenience. 

Sincerely,  

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Reviewer 1

Overall it was a great study, with human metastatic clear cell sarcoma sample being used to create a stable cell line containing characteristic chromosomal alterations. The authors did an outstanding work in characterizing the cell line and targeting the activated c-MET pathway by Pazopanib, a novel tyrosine kinase inhibitor.

Comments
1) Although the authors addressed Pazopanib's effect on CCS xenografts against c-MET using western blot, it would be more convincing to have immunohistochemical analysis done for HGF/c-MET and VEGF after the treatment.

Response to Reviewer comment No.1:
In accordance with reviewer's suggestion, we performed immunohistochemical analyses using MET, p-MET, and VEGF antibodies. We added the results of MET and p-MET in Supplementary Figure S5 and showed the results of MET, p-MET, and VEGF in Figure for reviewer 1.

Page 23, line 331, results
Supplementary information, page 7
Supplementary figure S5
Figure for reviewer 1

2) Although the original human CCS samples (supplementary figure 1) showed strong positivity for S-100, the xenografts were predominantly negative. It would be useful if the authors give a possible reason for the loss of S-100 immunoreactivity in xenografts in discussion section.

Response to reviewer comment No.2:
To reconfirm the immunoreactivity of the xenografts for S-100 protein, we used other two anti-S-100 antibodies for immunohistochemistry with freshly prepared specimens (IR50461; Dako, S2644; Sigma-Aldrich). As result, the xenografts showed strong positivity for S-100 protein with the both antibodies.
So we decided to change the Figure 2 and manuscripts as follows;
"Xenografts morphologically mimicked the primary tumor and expressed S-100 protein and antigens associated with melanin synthesis (Melan-A, HMB45)."
and
"The positive immunoreactivities of S-100 protein, Melan-A, and HMB45 in the Hewga-CCS xenografts were also similar to those of the primary tumor (Figure 2)."

Page 4, line 45-46, abstract
Page 19, line 269-271, results
Figure 2

3) A minor suggestion: Rephrasing the sentence in lines 279-281 is suggested for the readers benefit.

**Response to reviewer comment No.3:**

As the reviewer comments, the sentence in lines 279-281 and Figure 4 were confusing for the readers. We did SDS-PAGE again and obtained consistent figure.

We changed the Figure 4C and rewrote the sentence as follows;
"We then examined whether pazopanib-mediated c-MET inhibition affected intracellular signaling in Hewga-CCS cells. Decreases in Akt and Erk1/2 phosphorylation occurred concurrently with the decrease in c-MET phosphorylation (Figure 4C)."

Page 20-21, line 298-301, results
Figure 4C
Reviewer 2

In the current manuscript, the authors present the generation of a novel fusion gene-positive clear cell sarcoma and show data suggesting both potential for treatment with pazopanib (an agent that has been suggested to be of some utility in this family of tumors before) and show some data for the likely mechanism by which pazopanib, which is a multi-targeted agent exerts its effect. The paper is generally well-written and data is presented in a way that is very understandable. The observations are likely to be of interest for physicians caring for patients with this very rare disease and to researchers studying both multi-targeted RTKi's and clear cell sarcoma. There are, however, a number of questions raised by some decisions the authors have made that need to be addressed to make this paper acceptable for publication:

Comments
1) Generalizability and innovation. Probably the biggest weakness of this paper is a lack of data that would suggest that these findings could be generalized to other tumors, even to other people with CCS. This could easily be done by including other cell lines in both the in vitro and in vivo experiments. While the number of appropriate cell lines is small, even experiments with one or two other cell lines, especially including those which have Type I fusion genes would go a long way to making the generalizability of these finding more obvious. Alternatively/additionally, it would have been helpful to see why the investigators chose pazopanib specifically. Were there other agents that were less effective? Additionally, it would have been nice to see whether there was an improved response with combined therapies, such as those that have been used/suggested in clinical studies. While all of these things may be a lot to ask for a journal like BMC Cancer, some effort to generalize would be helpful.

Response to Reviewer comment No.1:

Unfortunately we do not have another CCS cell line. Instead, to make the generalizability of our findings that pazopanib could exert antitumor effects through the inhibition of HGF/c-MET signaling, we tried to examine the effect of pazopanib for sarcoma using Asra-Eps, which was our established human epithelioid sarcoma cell line driven by HGF/MET signaling. We found that pazopanib inhibited Asra-Eps cell growth in a dose-dependent manner, and
suppressed cell cycle progression through the inhibition of HGF/c-MET signaling (Figure 1 and 2 for reviewer 2).

We added the effects of pazopanib on Asra-Eps as follows;

“To examine whether pazopanib has the similar effects on other sarcoma cell line, we used Asra-Eps [29], which was our established epithelioid sarcoma cell line driven by HGF/c-MET signaling. We found that pazopanib inhibited Asra-Eps cell growth in vitro and autophosphorylation of c-MET in a dose-dependent manner (data not shown).”

The reason why we chose pazopanib specifically is that this drug has been approved for the treatment of advanced soft tissue sarcomas as the first molecular targeted agent in Japan. We added the sentence as follows;

“A phase III trial conducted to assess the efficacy and safety of pazopanib for metastatic STS using placebo as a control demonstrated a statistically significant improvement in progression-free survival [16], leading to approval of this drug for the treatment of advanced STSs as the first molecular targeted agent in Japan.”

Figure 1 and 2 for reviewer 2
Page 21, line 303-307, results
Page 6, line 87-88 background

2) Clinical course of patient. Since the paper suggests that the researchers jumped straight to evaluation of pazopanib for this tumor, it is interesting that there is no mention of the regimen with which the woman from whom this cell line was derived was treated. Specifically, there is no mention as to whether she received pazopanib. It would be important information to know if she had any kind of response to the agent. It would have been reasonable to think that someone with her clinical scenario would have been exposed to the drug.

Response to Reviewer comment No.2:

We appreciate your well-directed comments. In accordance with reviewer's comments, we added the chemotherapy regimen as follows;

“Despite of receiving local radiotherapy and three cycles of systemic chemotherapy composed of doxorubicin and ifosfamide,”

Since pazopanib had not been approved for sarcoma treatment in Japan at
that time, we did not use pazopanib for the patient. We added the explanation for pazopanib treatment as follows;

“Through the clinical course, the patient had not been exposed to pazopanib.”

Supplementary information, page 3, line 33-34
Supplementary information, page 3, line 37

3) Lack of survival studies. The authors show delay in growth for a short timeline with pazopanib. It would be generally accepted to also show survival benefit, and to extend the growth observations beyond 28 days. How long does the growth benefit last? This data would be very important to making inferences for clinical therapies. Ideally, the authors would show both growth inhibition and survival curves.

Response to Reviewer comment No.3:

We appreciate your helpful suggestion. However, according to the institutional animal care and use committee (IACUC) criteria, it is recommended to sacrifice the mouse before the burden tumor size has reached 20 mm. So we sacrificed the xenografted mice when the burden tumor size have reached approximately 20 mm and did not perform any survival studies.

4) In Figure 4C, there appear to be either problems or important unexplained phenomena. The p-MET timeline and p-Erk/AKT timelines don't match and show irregularities. There should be more than one sample per timepoint. If the patterns hold for multiple samples as shown in the figure, there should be some effort to suggest why p-MET activity is higher at 24 hours than at time 0, why pErk is going down when p-MET is going up, what happened to p-AKT at 6 hours, and why this matches so poorly with the in vivo data. This might be explained, at least partially, by reference to published literature, but no attempt is made. Again, this data would also be much stronger by limiting the number of timepoints and expanding the number of tumor lines.

Response to Reviewer comment No.4:
In accordance with reviewer's suggestion, we limited the number of
timepoints and use two samples per timepoint in Figure 4C. As a result, the
p-Erk/Akt timeline matched the p-MET timeline. We rewrote the manuscripts as
follows;

“We then examined whether pazopanib-mediated c-MET inhibition affected
intracellular signaling in Hewga-CCS cells. Decreases in Akt and Erk1/2
phosphorylation occurred concurrently with the decrease in c-MET
phosphorylation (Figure 4C).”

We also demonstrated the inhibition of c-MET signaling by pazopanib on
Asra-Eps cells (Figure 2 for reviewer 2).

Page 20-21, line 298-301, results
Figure 4C
Figure 2 for reviewer 2

5) In Figure 4a, the dot blot is grossly underexposed. Given this, it is difficult to
justify the inferences made. Also, it would be nice, with appropriate exposures,
to see treatment/non-treatment dot blots to see what pathways have changed.

Response to Reviewer comment No.5:
In accordance with reviewer's suggestion, we carried out RTK array analysis. In
spite of a longer exposure, we could not detected activation of VEGFR and
PDGFR (Figure 3 for reviewer 2). Thus, we did not change the Figure 4A.
Consistent with western blotting analysis (Figure 4B), phosphorylation of c-MET
was attenuated in Hewga-CCS cells treated with 10 µM pazopanib (Figure 3 for
reviewer 2).

Figure 3 for reviewer 2

6) Since the claim is made that the tumor recapitulates morphologically the
primary disease in the patient, the patient's micrographs should be shown
together with those from the xenograft model in Figure 2.

Response to Reviewer comment No.6:
In accordance with reviewer's comments, we showed the micrographs from xenografts and primary tumor side by side in Figure 2. In addition, we reconfirmed the positive immunoreactivity with different anti-S-100 antibodies on the freshly prepared specimens. Then we confirmed positive reactivity for S-100 in xenografts and changed Figure 2B and manuscripts as follows; "The positive immunoreactivities of S-100 protein, Melan-A, and HMB45 in the Hewga-CCS xenografts were also similar to those of the primary tumor (Figure 2)."

Page 19, line 269-271, results
Figure 2

7) Statistical testing for Figure 6 should utilize some type of repeated measures analysis--data are not independent.

**Response to Reviewer comment No.7:**

We used Fisher's exact test for statistical analyses. As a result, P value was changed from P<0.001 to P<0.05. We added the methods as follows; “The immunohistochemical results were statistically analyzed using Fisher's exact test.”

Page 44, line 659, figure legends
Page 16, line 234-235, methods

8) For the amount of work shown in the paper, the author list is awfully long. This may be appropriate, though the reviewer would ask the authors to ensure compliance with internationally-accepted standards for authorship.

**Response to Reviewer comment No.8:**

In accordance with reviewer's comments, we reduced 3 authors who had relatively less contribution to this report from the authors list.

Page 1, line 5-13, title
Reviewer 3

The manuscript is well written and authors did the outstanding work. The study is well designed. Data, Discussions and conclusions are good.

Comments
1) The author should consider to revised the title for better readability

Response to Reviewer comment No.1:
In accordance with reviewer's suggestion, we changed the title from "Establishment of Hewga-clear cell sarcoma, a new clear cell sarcoma cell line, and investigation of the antitumor effects of pazopanib on Hewga-CCS: an in vitro and in vivo study" to "Establishment of a novel clear cell sarcoma cell line (Hewga-CCS), and investigation of the antitumor effects of pazopanib on Hewga-CCS".

Page 1, line 1-3, title

2) First paragraph Background section -explain EWS

Response to Reviewer comment No.2:
We added the explanation of EWS as follows;
Ewing sarcoma gene (EWS).

Page 3, line 30-31, abstract

3) Third paragraph Result section -explain HMB-45

Response to Reviewer comment No.3:
We add the explanation of HMB45 in the sentence as follows;
"Xenografts morphologically mimicked the primary tumor and expressed S-100 protein and antigens associated with melanin synthesis (Melan-A, HMB45)."

Page 4, line 45-46, abstract
4) It is not clear why the authors want to develop this cell line, when other cell lines are available.

Response to Reviewer comment No.4:
To answer the reviewer’s comments, we added the explanation in abstract as follows;
“We established a novel CCS cell line called Hewga-CCS and developed an orthotopic tumor xenograft model to enable comprehensive bench-side investigation for intensive basic and preclinical research in CCS with a paucity of experimental cell lines.”

Page 3, line 32-35, abstract

5) First paragraph explain M-MITF, S-100 and HMB45.

Response to Reviewer comment No.5:
In accordance with reviewer’s comments, we add the explanation of M-MITF, S-100 and HMB45 as follows;
“Clear cell sarcoma (CCS) of tendons and aponeuroses is a rare, malignant, soft tissue tumor [1] characterized by melanocytic differentiation, including immunohistochemical positivity for melanocyte specific-microphthalmia-associated transcription factor (M-MITF), S100 calcium binding protein (S-100), Melan-A, and melanoma-associated antigen human melanoma black 45 (HMB45).”

Page 5, line 59-63, background

6) First paragraph- Please provide the reference for approximately "50% patients develop nodal metastases"

Response to Reviewer comment No.6:
In accordance with reviewer’s comments, we add the reference for the
sentence as follows;
“It usually appears as a deep-seated, slowly growing mass, and approximately 50% patients develop lung or nodal metastases [2].”

Page 5, line 65-66, background

7) First paragraph-Please rephrase the sentence "CCS is molecularly defined resulting in the EWSR1-ATF1 (EWS-ATF1) fusion oncoprotein [12, 13]."

Response to Reviewer comment No.7:
In accordance with reviewer's suggestion, we rewrote the sentence as follows;
"Cytogenetic analysis of CCS has detected the presence of clonal chromosomal translocation, t(12;22)(q13;q12), and identified the fusion of the ATF1 and EWS, resulting in the EWS-ATF1 fusion gene [12,13]."

Page 5, line 68-71, background

8) Paragraph 2- It is not clear why this Hewga-CCS cell line is developed when other cell lines are available to study CCS. Authors should also tried to explore the effect of Pazopanib in other available cell lines.

Response to Reviewer comment No.8:
In accordance with reviewer's comments, we add the sentence as follows;
Page 7, line 90-93, background
"To date, a small number of CCS cell lines have been successfully established [17-27], but those harboring disease specific EWS-ATF1 fusion gene and available in both in vitro and in vivo study are quite rare. Thus, we established a new CCS cell line, Hewga-CCS, and investigated the antitumor effects of pazopanib on Hewga-CCS in vitro and in vivo."
We also explain the significance of establishment of Hewga-CCS in discussion;
there are only 4 cell lines that have been shown to possess both tumorigenicity in immunodeficient mice and EWS-ATF1 fusion transcripts (Table 1). Furthermore, there is only 1 cell line (UM-CCS-1) that has the type 2 EWS-ATF1 transcript (Table 1). However, UM-CCS-1 could be passaged only in nude mice. Therefore, Hewga-CCS is the first cell line that harbors the type 2 chimeric EWS-ATF1 transcript and can be stably cultured in vitro and xenografted in nude mice.

Unfortunately we do not have another CCS cell line. Instead of using another CCS cell line, we examined the effect of pazopanib for sarcoma using Asra-Eps, which was our established human epithelioid sarcoma cell line driven by HGF/MET signaling. Pazopanib inhibited Asra-Eps cell growth in a dose-dependent manner, and suppressed cell cycle progression through the inhibition of HGF/c-MET signaling. (Figure 1 and 2 for reviewer 3).

We added the effects of pazopanib on Asra-Eps as follows:

To examine whether pazopanib has the similar effects on other sarcoma cell line, we used Asra-Eps [29], which was our established epithelioid sarcoma cell line driven by HGF/c-MET signaling. We found that pazopanib inhibited Asra-Eps cell growth in vitro and autophosphorylation of c-MET in a dose-dependent manner (data not shown).

9) Chromosomal analysis- Please explain the method in detail.

Response to Reviewer comment No.9: We added the method of chromosomal analysis as follows; Metaphase chromosome spreads from Hewga-CCS cells were prepared according to standard procedures. Hewga-CCS cells were treated with 20 µg/ml of colcemide overnight and harvested. After treatment of 0.075 M KCl for 20 min at 37°C, cells were fixed 3 times with methanol and acetic acid (3:1) and
fixed cells were spread on slides. Multicolor fluorescence in situ hybridization (M-FISH) was performed using commercially available M-FISH kits (MetaSystems, Altlussheim, Baden-Württemberg, Germany) according to the manufacturer’s protocol. Briefly metaphase spreads were hardened 70°C for 2 h. After applying M-FISH probes on the metaphase spreads, co-denaturation of target DNA with probe DNA was performed at 70°C for 5 min, followed by 72 h incubation at 37°C to allow hybridization of the probes. The slides were then washed twice with 50% formamide/2 × standard saline citrate (SSC) solution for 20 min at 37°C, 2 × SSC for 10 min at room temperature and 1 × SSC for 10 min. The slides were then counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and mounted. Separate fluorochrome images were captured using a Leica DC 350FX cooled CCD camera (Leica Microsystems, Wetzlar, Hesse, Germany) mounted on a Leica DM600 B microscope using Leica DM600 B software. The images were analyzed using Leica CytoVision (Leica). The chromosomal analyses were examined at passage 110 and 111.”

Page 8-9, line 115-132, methods

10) Cell proliferation assay- Please provide the passage number of the Hewga-CCS cells used

Response to Reviewer comment No.10:
We added the information of the passage number in the methods as follows;
“These analyses were examined at passage 120 to 130.”

Page 11, line 162-163, methods

11) Characterization of the Hewga-CCS cell line- It is not clear why 20% FBS is used for spheroid formation.

Response to Reviewer comment No.11:
Spheroid formation was usually seen in the serum free medium with
proper mitogens such as bFGF and EGF. However, we previously reported that spheroid formation was observed even when replated in the serum-containing medium (DMEM with 20% FBS) on the low attachment plates (Naka et al. Stem Cells 2010;28:1119-1131). We add the reference for the explanation as follows; “To examine the capacity of spheroid formation, we cultured the cells on low-attachment dishes with 20% FBS according to the protocol of our previous study [28].”

Page 17, line 243-245, results
Reviewer 4

In this work the authors have established a novel Hewga-CCS cell line and developed a xenograft mouse model. Subsequently they described the antitumor effects of Pazopanib via inhibition of HGF/c-MET signaling in these cells. The study is well planned and executed. The findings may be important, and have potential to represent a unique contribution to the field with broader implications for cancer chemoprevention. The paper will be of interest to a wide audience.
Reviewer 5

This is a study which details establishment of a Clear cell sarcoma (CCS) cell line (Hewga-CCS) that has developed a model to enable comprehensive bench-side investigation. This is a good study and should be published. However, there are some concerns as follows that in my opinion should be addressed before the manuscript is accepted for publication.

Comments
1) Pg-8: Beginning: How many times the chromosomal analysis of the cells was performed in the span of 36 months? At what passage numbers? The authors may be asked to mention in the MSS……

Response to Reviewer comment No.1:
We add the information of the passage number in the methods as follows;
"The chromosomal analyses were examined at passage 110 and 111."

Page 9, line 131-132, methods

2) Pg-8 Beginning: the attached cells continuously expressed the EWS-ATF1 transcript (data not shown). The authors have measured EWS-ATF1 transcript. What was the state with respect to ATF-2 and ATF-3 transcripts? (the authors have mentioned the same in introduction) The authors may be asked to mention in the MSS……

Response to Reviewer comment No.2:
We appreciate your kind comments for our confusing part of splice variants in the EWS-ATF1 fusion gene.

The EWS-ATF1 has 3 splice variants (type 1~3). Detail of the variants was written in the manuscript at page 5-6, line 71-74;
"Several types of fusion transcripts have been described, of which the most common result from the fusion of exon 8 of EWS with exon 4 of ATF1 (type 1), followed by the fusion of exon 7 of EWS with exon 5 of ATF1 (type 2) and the fusion of exon 10 of EWS with exon 5 of ATF1 (type 3) [14]."
Hewga-CCS has type 2 splice variant of *EWS-ATF1* as shown in Figure 1D. Because *ATF2* and *ATF3* have no relation to chimeric fusion gene of CCS, we did not refer to these genes.

3) Methods: Pg. 8 beginning: The authors mention “Throughout the establishment of this cell line, the attached cells continuously expressed the EWS-ATF1 transcript (data not shown).” In Genetic analysis: (Pg. 9) EWS-ATF1 cDNA was identified by polymerase chain reaction (PCR) using EWS forward primer 5’-TCC TAC AGC CAA GCT CCA AGT C and ATF1 reverse primer 5’-ACT CGG TTT TCC AGG CAT TTC AC. The Authors performed direct sequencing of the PCR product. Results: Figure 1D shows that the Type 2 control matches at 464 bp in Hewga-CCS cells. Figure 1E shows that the authors obtained Type 2 fusion transcript. Thus, the authors could get type 2 fusion transcript. This is contrary to the one mentioned in Methods. The authors therefore may be asked to bring the same in the MSS at all appropriate locations.

**Response to Reviewer comment No.3:**

As mentioned above, we detected *EWS-ATF1* chimeric fusion gene from Hewga-CCS and confirmed the *EWS-ATF1* of Hewga-CCS as type 2 splice variant.