Author's response to reviews

Title: Peretinoin, an acyclic retinoid, improves the hepatic gene signature of chronic hepatitis C following curative therapy of hepatocellular carcinoma

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Author's response to reviews: see over
Dear Prof. Hummel,

Re: MS 9573968280535457, “Peretinoin, an acyclic retinoid, improves the hepatic gene signature of chronic hepatitis C following curative therapy of hepatocellular carcinoma” by Masao Honda, Taro Yamashita, Tatsuya Yamashita, Kuniaki Arai, Yoshio Sakai, Akito Sakai, Mikiko Nakamura, Eishiro Mizukoshi and Shuichi Kaneko

Thank you for the constructive and thoughtful comments regarding our manuscript. We have tried to answer all of the comments raised by the reviewers and have revised the manuscript accordingly. Our detailed point-by-point responses follow this letter. We believe that our study is now more scientifically sound and hope that you and the reviewers will find our revised manuscript acceptable and that the paper meets the editorial requirements.

We look forward to hearing from you.

Yours sincerely,

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Associate Editor's comment:

Although the authors provided interesting data, several aspects have to be considered. Since there was no detectable peretinoin level in the liver, the authors draw an indirect conclusion from the gene expression data that sufficient amount of peretinoin reached the liver cells. Moreover the discrimination of treatments with 300 mg and 600 mg is based on the unknown concentration in the liver - at least questionable. The gene expression data produced derive from non-tumor liver specimens of only peretinoin treated patients. This raises the question regarding gene expression differences in patients without peretinoin treatment over time. I am confused by the statement (p 16) that "We argue that peretinoin suppresses HCC cell proliferation by improving these genetic abnormalities, thereby preventing HCC recurrence." What is meant by "improving these genetic abnormalities"?

As pointed, plasma concentrations of the lipid-bound form of peretinoin were dose-dependent, while liver peretinoin concentrations in patients were below the detection limit. Multiple factors might be associated with the metabolism of peretinoin in the liver, including the lipid content and the degree of liver function (oxidation and TCA cycle in mitochondria). Therefore, we were unable to monitor peretinoin concentrations in the liver in this study. On the other hand, plasma concentrations of the lipid-bound form of peretinoin were dose-dependent, assuming a dose-dependent exposure of peretinoin to the liver. In fact, peretinoin-response genes were more strongly induced in response to a 600 mg dose of peretinoin. We added a sentence to the revised manuscript with respect to this: “Serial changes in peretinoin-responsive gene expression are shown in Supplemental Fig. 1. Significant changes in expression were observed in response to 600 mg of peretinoin, while changes in expression were minimal with 300 mg of peretinoin” (page 12, line 25 and page 13 lines 1-3).

It was pointed that gene expression differences in patients without peretinoin treatment over time were not evaluated. We explained this in the revised manuscript: “In addition, we did not conduct a placebo control to observe serial changes of hepatic gene expression without peretinoin administration. Therefore, there might be some limitations in drawing concrete conclusions from this study” (page18, lines 21–23).

The meaning of “genetic abnormalities” was questioned. In the revised manuscript, we changed “genetic abnormalities” to “the expression of these genes” (page 17, line 8).
The study of Honda et al was designed for determining the molecular basis of the beneficial effects observed in a previously performed phase II/III clinical trial for peretinoin maintenance treatment after curative treatment of hepatocellular cancer patients. Liver biopsies from 12 HCC patients who had been treated with curative resection or ablation were taken at baseline and week 8 of peretinoin treatment. Biopsies were subjected to gene expression analysis of mRNA levels. The predictive value of gene expression pattern for recurrence was assessed. The rationale of the study is clearly defined and the experiments were well performed. The authors should be congratulated for their molecular translational study with clinical samples collected before and under treatment. Nonetheless, there are some critical issues which should be addressed before the manuscript would be acceptable for publication.

We thank the reviewer for such encouraging comments.

**Major compulsory revisions:**

1) As mentioned in the Introduction, the authors have performed a previous multicenter, double-blinded Phase II/III study in which 401 HCC patients have been randomized to receive peretinoin at a dose of 300mg or 600mg or a placebo. The results of this previous study have not been reported in a full paper, but preliminary efficacy data were presented at ASCO 2010. As mentioned in the ASCO abstract, recurrence-free survival after 2 years was significantly improved in the patient group receiving 600 mg per day compared to placebo (HR 0.27; 0.07-0.96). However, a daily dose of 300 mg peretinoin did not improve but rather slightly increased the recurrence risk when compared to placebo (HR 1.19, 0.55-2.60).

In their current study, the authors evaluated the molecular response of liver cells to peretinoin treatment again in a 300-mg and 600-mg dose group. However, the authors did not separately report on the expression profile analysis from these two dose groups and it remains unclear why they have chosen two different concentrations. Considering the different outcome reported before, it would be
interesting to know whether or not differences were also observed on the molecular level. Since only a very small number of patients was included in the molecular pharmacokinetic study the rationale for choosing two different concentrations should be given.

The reviewer has made some fair points. The multicenter, double-blinded Phase II/III study of 401 HCC patients started in February 2005 and the current clinical pharmacology study started in June 2006. Before starting the clinical pharmacology study, we did not know the results of the phase II/III study and expected that a 300 mg dose of peretinoin would be effective. For this reason, we planned to observe a dose escalation effect from 300 mg to 600 mg, which is why we set two different doses of peretinoin in this study. As the reviewer pointed out, considering the different outcomes of the two doses, it would have been better to analyze the 300 mg dose and 600 mg dose separately. Therefore, we did this in the revised version of the manuscript. We identified peretinoin-response genes by comparing hepatic gene expression in 6 patients pre-treatment and during treatment who received a 600 mg dose of peretinoin. We modified Table 2 and showed the fold changes of gene expression following a 300 mg and 600 mg dosage. We also analyzed the serial changes of peretinoin-response genes and represented these in Supplemental Fig.1. We added the following sentence to the revised manuscript: “The phase II/III clinical study showed that a daily dose of 600 mg peretinoin reduced the risk of HCC recurrence, while a 300 mg daily dose was not significantly different from the placebo [17]. Therefore, gene expression patterns were compared before and after the start of the 600 mg peretinoin therapy (n = 6). Consequently, 424 hepatic genes showed significantly different expression levels from baseline at week 8 (enhancement and suppression seen for 190 and 234 genes, respectively). Typical examples of these genes are represented in Table 2 where fold changes of gene expression for the 300 mg and 600 mg doses are shown respectively” (page 12, lines 13–20). We also added this sentence: “Serial changes in peretinoin-responsive gene expression are shown in Supplemental Fig. 1. Significant changes in expression were observed in response to 600 mg of peretinoin, while changes in expression were minimal with 300 mg of peretinoin” (page 12, line 25 and page 13 lines 1-3).

Regarding peretinoin-response genes, we added the following description: “Interestingly, 44 out of 224 (20%) genes were peretinoin induced” (page 13, lines 21–22). We also added the following description: “Supervised learning methods identified 224 genes as predictors for HCC recurrence (p<0.002). Importantly, 44 of these (20%)
genes were peretinoin-responsive genes, suggesting that recurrence-related genes might be regulated by peretinoin-responsive genes.” (page 17, lines 11–14).

2) It remained also unclear to the reviewer whether or not the results from expression profiling of the 300-mg group was also included in the model for identification of a predictive gene signature. Given that the subsequent maintenance treatment after the experimental 8-week start phase consisted of 600 mg peretinoin for all patients the inclusion of the expression profiles from the 300-mg group in the class prediction might represent a significant bias in their model. This is important since the authors conclude from their study that response assessment during the early period of administration might predict recurrence-free survival. The authors should explain their procedure for the hierarchical clustering, especially in terms of which samples were included, and should also discuss more in detail potential biases in this analysis.

The reviewer raises some fair points. For the prediction analysis, we included all 12 patients. Gene expression profiling in the liver 8 weeks after peretinoin treatment was significantly classified into two groups of HCC recurrence and non-recurrence, but was not classified into groups of 300 mg and 600 mg dosages as demonstrated in Table 3. 224 genes were obtained as predictors for HCC recurrence, while only 38 genes were obtained as predictors for dosage. Therefore, gene expression profiling in the liver 8 weeks after peretinoin treatment was more related to HCC recurrence than dosage. One possible reason for this is that some patients receiving a 300 mg dose had already expressed high levels of peretinoin-response genes before starting peretinoin treatment. We added the following sentence to the revised manuscript: “Although peretinoin-responsive genes were more induced in patients treated with the 600 mg dosage, gene expression profiling 8 weeks after peretinoin treatment could not be classified according to dosage (Table 3). This might be because 2 patients treated with the 300 mg dosage (No. 11 and No. 12) had already expressed high levels of peretinoin-response genes before starting peretinoin treatment (Supplemental Fig. 1). Interestingly, patients with high levels of peretinoin-response genes before treatment (No. 9–12) did not show HCC recurrence during the entire observation period (4.5 years; Table 1)” (page 13, lines 14–21).

As the reviewer pointed out, the subsequent maintenance treatment after the experimental 8-week start phase consisted of 600 mg peretinoin for all patients. Thus,
inclusion of the expression profiles from the 300-mg group in the class prediction might represent a bias in our model. We added the following sentence to explain this: “This study demonstrated that the patient response to peretinoin during the early period of administration could predict HCC recurrence and, potentially, patient survival. However, it should be noted that the current study protocol consisted of 600 mg peretinoin as the subsequent maintenance treatment for all patients after the 8-week start phase (Fig. 1A). In addition, we did not conduct a placebo control to observe serial changes of hepatic gene expression without peretinoin administration. Therefore, there might be some limitations in drawing concrete conclusions from this study” (page 18, lines 17–23).

3) As stated on page 6 all patients were clinically tumor-free which means that the gene expression profiles were generated from normal liver tissue. Even if the presence of putative tumor stem cells or residual tumor cells cannot be excluded the molecular pattern mainly represents the non-tumor cell fraction. The authors should discuss in more detail how the expression profile in normal liver cells might determine the recurrence risk? Did the authors also evaluate the expression profiles at the time point when the tumor recurred in order to see whether the same signaling pathways were still activated?

This is an important question but one that is difficult to answer fully. As we did not evaluate the expression profiles at the time point when the tumor recurred, we could not discuss the serial changes in gene expression in tumor-free and tumor-recurrent cases. The detailed mechanism of how the expression profile of non-tumor liver tissues might determine the recurrence risk is not known at present. However, we speculate that peretinoin directly represses HCC through peretinoin-response genes and indirectly suppresses HCC recurrence through recurrence-related genes. The expression of these genes was clearly shown in non-tumor liver tissues in this study.

We added the following explanatory sentence: “The exact mechanisms of how the expression profile of non-tumor tissues might determine the recurrence risk are not known. However, the degree of differentiation of hepatocytes and microenvironments such as angiogenesis and fibrogenesis in non-tumor lesions of the liver is likely to be closely associated with hepatocarcinogenesis. Interestingly, patients with pre-activated peretinoin-response genes were resistant to HCC recurrence during the entire observation period (4.5 years)” (page 18, lines 11–16).
4) The rationale for selecting week 8 for the second biopsy should be given. How can the authors rule out that an earlier assessment of gene expression would not be superior for predicting the beneficial peretinoin effect?

From the pharmacokinetics phase I study, the concentration of the lipid form of peretinoin in plasma stabilized 2 weeks after starting the peretinoin therapy. This concentration was maintained for 24 weeks through repeated doses of peretinoin. Therefore, a 2-week interval for the second liver biopsy would be possible from the pharmacokinetics viewpoint of peretinoin. However, to minimize the stress of repeated liver biopsies for the patients, we set the interval at 8 weeks.

5) Liver peretinoin concentrations in 10 of 12 patients were below the detection limit and, as discussed by the authors on page 17 the analysis of a possible relationship between peretinoin concentrations and the molecular expression profiles was not possible. In contrast, plasma concentrations were detectable and dose-dependent. Was there a dose-relationship between plasma levels and expression profiles? If not, why did the authors include these data here?

As the reviewer pointed out, plasma concentrations of the lipid-bound form of peretinoin were dose-dependent, while liver peretinoin concentrations in patients were below the detection limit. Multiple factors might be associated with the metabolism of peretinoin, for example, the lipid content of the liver and the degree of liver function (oxidation and TCA cycle in mitochondria). Therefore, we were unable to monitor the peretinoin concentration in the liver in this study. On the other hand, plasma concentrations of the lipid-bound form of peretinoin were dose-dependent, assuming a dose-dependent exposure of peretinoin to the liver. In fact, peretinoin-response genes were more strongly induced following a 600 mg dose of peretinoin. We described this in the revised manuscript as: “Serial changes in peretinoin-responsive gene expression are shown in Supplemental Fig. 1. Significant changes in expression were observed in response to 600 mg of peretinoin, while changes in expression were minimal with 300 mg of peretinoin” (page 12, line 25 and page 13 lines 1-3).
6) As seen in Table 1, 5 of 6 patients with recurrences at 4.5 years were female and only 1 of 5 males experienced a tumor recurrence at that time. Did the authors observe a difference in the expression profiles in females and males? Is there evidence that the action of peretinoin might differ depending on the gender?

As the reviewer pointed out, more of the female patients had HCC recurrence. However, this might be because of the small number of patients enrolled in this study as we did not observe sex differences in HCC recurrence in a large-scale phase II/III study.

**Reviewer 2**
Dr. Masahito Shimizu

This manuscript from Dr. Honda M. et al. describes the detailed mechanisms of peretinoin for suppressing HCC recurrence based on the gene expression profiles. Their findings are novel and significant. The authors’ experiments appear to be technically solid and their data are convincing. The text is also well written and I have no serious criticism regarding methodology, results, and interpretation of results.

We thank the reviewer for the encouraging comments.

**Editorial request(s):**

1) Requesting deposition of data:

Nucleic acid sequences, protein sequences, and atomic coordinates should be deposited in an appropriate database in time for the accession number to be included in the published article. In computational studies where the sequence information is unacceptable for inclusion in databases because of lack of experimental validation, the sequences must be published as an additional file with the article. Where appropriate, authors should adhere to the standards proposed by the Microarray Gene Expression
Data Society (http://www.mged.org) and must deposit microarray data in MIAME-compliant format in one of the public repositories, such as ArrayExpress (http://www.ebi.ac.uk/arrayexpress), Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/projects/geo/) or the Center for Information Biology Gene Expression Database (CIBEX; http://cibex.nig.ac.jp).

We added the following sentence to the revised manuscript: “The microarray data have been submitted to the Gene Expression Omnibus (GEO) public database at NCBI (Accession No. GSE29302)” (page 10, lines 12–13).

2) PLEASE include TRN below Abstract section: The Abstract of the manuscript should not exceed 350 words and must be structured into separate sections: Background, the context and purpose of the study; Methods, how the study was performed and statistical tests used; Results, the main findings; Conclusions, brief summary and potential implications. Please minimize the use of abbreviations and do not cite references in the abstract. TRIAL REGISTRATION, if your research article reports the results of a controlled health care intervention, please list your trial registry, along with the unique identifying number (e.g. Trial registration: Current Controlled Trials ISRCTN73824458). Please note that there should be no space between the letters and numbers of your trial registration number. We recommend manuscripts that report randomized controlled trials follow the CONSORT extension for abstracts.

We described the registration of this trial: “The study was registered at the Japan Pharmaceutical Information Center (JapicCTI-121757)” (page 8, line 15-16).