［別紙２］

審査の結果の要旨

氏名　　ナラヤン　ダレル

本研究では、癌抑制遺伝子である p53 の C 型肝炎ウイルス複製に対する影響を評価するため、p53 を knock down あるいは強制発現した細胞株を作成し、subgenomic replicon RNA、full-length HCV RNA の増殖効率を比較検討した。そして、以下の結果を得ている。

1）control 細胞株に比し p53 の knock down 細胞株では、subgenomic replicon RNA、full-length HCV RNA いずれにおいても増殖効率の上昇を認め、逆に p53 の強制発現細胞株では増殖効率の低下を認めた。これより、p53 は HCV 感染に対する宿主の防御機構において、重要な役割を果たしていることが示唆された。
2）免疫沈降法により、p53 が IRF9 と結合することを証明し、さらに p53 の knock down Huh7 ではインターフェロン添加によるインターフェロン誘導遺伝子の活性化が減弱することを明らかにした。すなわち、p53 の HCV に対する増殖抑制は、IRF9 との結合を介するインターフェロン誘導遺伝子の活性化によるものと推察された。
3）p53 の N 端もしくは C 端を欠如した deletion mutant p53 を作成し、subgenomic replicon RNA の複製に対する影響を評価したところ、regulatory domain である C 端を欠如した p53 では wild type の p53 と同様の HCV 増殖抑制が認められたが、*trans*-activation domain である N 端を欠如した p53 では HCV 増殖抑制が認められなかった。すなわち、p53 の HCV 増殖抑制作用には、N 端側の *trans*-activation domain が重要であると考えられた。

以上、本論文により、p53 は HCV 感染に対する宿主の防御機構において、重要な役割を果たしていることが示唆された。また、p53 の HCV 増殖抑制作用は、IRF9 との結合を介するインターフェロン誘導遺伝子の活性化によるものと推察された。p53 の HCV 増殖に対する直接的な影響については、本研究が初の報告と思われ、学位の授与に値すると考えら

れる。
尚、審査会時点から、論文の内容について以下の点が改訂された。

Major Comments:

Comment 1. Change the title.

As suggested by the jury, the title has been changed to:

"Potential Contribution of Tumor Suppressor p53 in the Regulation of Hepatitis C Virus Replication"

C 型肝炎ウイルス複製の制御における癌抑制遺伝子 P53 の関与について

Comment 2. Explain fully the status of p53 in Huh7 cells.

The status of p53 in Huh7 cells is explained in detail. The following paragraphs have been added in the dissertation in the **Discussion section, page 48, 1st paragraph:**

"It is important to note that the Huh7 cells actually harbors a Y220C mutant p53 rather than the wild type (31, 38) which could possibly compromise the *in-vivo* significance of our experimental results. However, despite the gene mutation, Huh7 cells are known to maintain an effective p53 *trans*-activation activity comparable to that of Chang liver cells and HepG2 cells, both of which contain wild type p53 (39-42). The cell growth inhibition and apoptotic cell death patterns of Huh7 cells are comparable with HepG2, but not with Hep3B cells which is p53 deficient (40, 41). We tested the activity of the Y220C mutant p53 which was found to retain *trans*-activational activity and was able to suppress HCV RNA replication in Huh7 cells (Fig. 6b and 6c) further supporting the notion that the Y220C mutant p53 in Huh7 cells is functionally intact."

Following references were also included in the reference section.

38. Hsu IC, Tokiwa T, Bennett W, Metcalf RA, Welsh JA, Sun T, Harris CC. p53 gene mutation and integrated hepatitis B viral DNA sequences in human liver cancer cell lines.Carcinogenesis.1993;14:987-992

39. Hsieh JL, Wu CL, Lee C H, and Shiau A L. Hepatitis B virus X protein sensitizes hepatocellular carcinoma cells to cytolysis induced by E1B-deleted adenovirus through the disruption of p53 function. Clin Cancer Res 2003; 9: 338-345.

40. Chiba T, Yokosuka O, Arai M, Tada M, Fukai K, Imazeki F, Kato M, Seki N, and Saisho H. Identification of genes up-regulated by histone deacetylase inhibition with cDNA microarray and exploration of epigenetic alterations on hepatoma cells. J Hepatol 2004; 41, 436-445.

41. Terui T, Murakami K, Takimoto R, Takahashi M, Takada K, Murakami T, Minami S, Matsunaga T, Takayama T, Kato J, Niitsu Y. Induction of PIG3 and NOXA through acetylation of p53 at 320 and 373 lysine residues as a mechanism for apoptotic cell death by histone deacetylase inhibitors. Cancer Res 2003;6:8948-8954.

Comment 3. Clarify figure 1b. Mention about the clones whenever necessary.

The Fig. 1b is slightly modified with new labeling and numbering of the p53kd clones. These Huh7 cells were obtained from Dr. N Sakamoto (Tokyo Medical Dental University, Tokyo, Japan). For preparation of stable p53 knocked-down Huh7 cells, these cells were transfected with siRNA-p53 vector and selected with hygromycin. The control Huh7 cells, right most panel (Fig. 1b), is transfected with the negative control

siRNA vector.

All of the experiments with comparison between control and p53kd Huh7 cells are performed using the control Huh7 (transfected with the negative control siRNA, right most lane, Fig. 1b) and the p53kd Huh7 clone 3 (lane 4, Fig. 1b). Unless specified otherwise, the p53kd Huh7 cell refers to the clone 3 throughout this report. The only experiment which uses another clone of p53kd Huh7 cell is the Fig. 1d, where the p53kd clone 1, was used together with p53kd Huh7 clone 3 for comparison. I sincerely apologize for making confusions during the presentation by putting a wrong figure.

In order to clarify this point, the following text is added in the results section.

Results section, page 20, line 6:

"As shown by the Western blot (Fig. 1b), out of the 5 clones of Huh7 cells transfected with the siRNA-p53, p53 knock down was most effective on the clone 3. In our subsequent experiments, unless specified otherwise, we used the p53kd Huh7 clone 3 as the p53kd Huh7 and the Huh7 cells transfected with the negative control siRNA (right most lane, Fig. 1b), as the control throughout our experiments.

Following correction was made in the Figure 1 legends:

Figure 1 Legends (b) in page 25:

"Establishment of stable p53 knocked down cells. Huh7 cells were transfected with siRNA-p53 vector or the negative control siRNA, selected with hygromycin for 3–4 weeks and p53 protein expression was checked by Western blotting. The p53kd Huh7 clone 3 (lane 4) was used as the p53kd Huh7 cell and the Huh7 cells transfected with the negative control siRNA (right most lane) was used as the control in our subsequent experiments."

Comment 4. In regards to figure 1d, repeat the experiment with proper control.

The experiment has been repeated with proper control as advised by the jury and the results are presented in the new Fig. 1d. The control Huh7, p53kd Huh7 clone 1, and p53kd Huh7 clone 3 were transfected with the Neo HCV replicon, and Western blot analysis of p53 and NS5B protein, and real time RT PCR quantification of the HCV RNA copy were performed as described in the methods section.

The following text is added in the result section.

Result 4.1, page 20, last line:

"Next, we took the control, p53kd Huh7 clone 1 with a partial p53 knock down, and the p53kd Huh7 clone 3 with almost complete p53 knock down, and transfected them with the Neo replicon. We evaluated the amount of HCV RNA by real time RT PCR, and NS5B protein by Western blot, which were clearly higher in the p53kd Huh7 cells than in the control, indicating that p53kd Huh7 cells are more permissive for the Neo replicon (Fig. 1d)."

Following correction was made in the **Figure 1 Legend (d), Page 25:**

(d) "Control Huh7 and p53kd- clone 1 and clone 3 cells were transfected with the Neo replicon and cell lysates were compared for the p53 and HCV NS5B by Western blot and for the replicon RNA copies by real-time RT-PCR"

Comment 6. You must explain why cell lines Skhep1 and Huh6 were not permissive for the HCV replicons.

In order to clarify this point, the following text is added in the discussion.

Discussion, page 48, 2nd paragraph:

"Huh7 cells are the only cell line known to support the HCV replication *in-vitro* consistently and persistently and are widely used for HCV cell culture (42, 43). For reasons still to be clarified, other human hepatoma cell lines including SKHep1, HepG2, Huh6, which harbor a wild type p53 are generally not permissive for the HCV replication. We, therefore, had to limit our study with the Huh7 cells alone. In fact, we tried to verify our experimental findings that p53 gene knock down makes the cells more permissive for the HCV replication using Huh6 and the SKHep1 cells, which in deed, supported our hypothesis by permitting some degree of HCV replication upon p53 gene knock down (Fig 1h and 2d). These assays, however, were transient assays. It will be more informative if the details of HCV replication profile could be tested in a cell harboring a wild type p53. On the other hand, cell lines such as Hep3B which are p53 deficient, are not permissive for the HCV replication. Hence, the mechanisms regulating the permissiveness for the HCV replication are complex and poorly understood. None the less, p53 seems to be one of the key players in determining the replication efficiency of HCV in the Huh7 cells."

The following references are added in the reference section.

42. Bartenschlager R, Kaul A, Sparacio S. Replication of the hepatitis C virus in cell culture. Antiviral Res 2003;60:91-102.

43. Pietschmann T, Bartenschlager R.Tissue culture and animal models for hepatitis C virus. Clin Liver Dis 2003;7:23-43.

Comment 7. In figure 5c, include the control. You may use controls like cmv-luc or NF-kB luc.

We repeated this experiment using the NFkB-Luc and the results are presented in the Figure 5d.

Following text is added.

Result 4.6, Page 38, Line 19.

"As a control, we evaluated the activation of the NFkB-Luc as well. As shown in the figure, there was no activation of the NFkB promoter by IFN in both the control and the p53kd Huh7 cells (Fig. 5d)."

Following legend is added in the **Figure legends 5d, page 38.**

"(d) Control and p53kd Huh7 cells were transfected with NFkB-Luc and pRL-TK, and luciferase assay was performed exactly as in (c)."

Comment 8. Interpret very carefully in terms of the generalizability in other mammalian cells and *in-vitro* vs. *in-vivo* application of the experimental findings.

Thank you for pointing out this very important point. The following change has been made in the **discussion section page 49, last line:**

"Though these findings are intriguing, the *in-vivo* significance of our *in-vitro* findings is unknown at this point. A careful interpretation of these *in-vitro* findings and verification by further experiments including animal models would be more informative in this regard."

Comment 9. In figure 3, there seems no evidence that p53 is working in Huh7 cells. You must show p53 inducible gene is expressed by p53 transfection.

As suggested by the reviewer, we have performed Western blot analysis of p21 in Huh7 cells following pCXN2-p53, siRNA-p53 or the empty vector transfection in the

Huh7 cells, and the result is included as Fig. 3d.

The following text is added in the result section.

Result 4.3, page 32, line 18:

"To confirm that transfected pCXN2p53 or the siRNAp53 in the Huh7 cells are actually functioning, we performed western blotting of the p21 which is one of the most important p53 downstream genes. As shown in the figure, p53 overexpression led to upregulation of the p21 protein where as p53 gene knockdown suppressed p21 expression (Fig. 3d)."

The following Figure Legends was added:

Figure legends 3(d), page 32:

"(d) p21 expression in the Huh7 cells transfected with empty vector, pCXN2-p53 or siRNA-p53 was checked by Western blotting after 48 hours of transfection."

Minor points

In page 28 and page 30, there is doubling of the same sentence. This is redundant.

I sincerely apologize for this error. The redundant part of the paragraph has been deleted.

Replace the word naïve with control throughout the report.

Thank you very much for your important suggestion. The word “naïve” have been replaced with “control” throughout the report.

In page 55, the reference 10 is erroneously printed.

I sincerely apologize for the error. Thank you very much for carefully reviewing the dissertation. I removed this error.

Finally, it is advised that interferon response in other cell line in which p53 is mutated be tested.

Thank you once again for your critical suggestions. I tested the interferon response in the HepG2 and Hep3B cells essentially as described in the methods section. HepG2 was chosen as it harbors wild type p53 and Hep3B is constituently p53 deficient. The result is included in the Fig. 5f.

The following text has been added in the result section:

Result 4.6, page 39, line 7:

“To corroborate our findings, we checked the expression of PKR, OAS, MxA and IRF7 mRNA in the HepG2 and Hep3B cells as well, the former containing a wild type p53 and the latter being p53 deficient (Fig. 5f). Interestingly, there was no induction of these ISGs by IFN in the Hep3B cells, where as HepG2 cells showed

significant levels of ISG induction following IFN treatment."

The following figure legend is added in the **Figure 5 Legend (f), in page 38:**

"(f) HepG2 and Hep3B cells were plated on the 60 mm culture dish, treated with or without 100 U/ml of IFN and real time RT PCR quantification of the PKR, MxA, OAS, and IRF7 mRNAs was performed exactly as described in (e)."