Note

Evaluation of miR-122 to Predict High Dose Acetaminophen-Induced Liver Injury in Mice: The Combination Uses of 5-Fluorouracil

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Administration of high doses of acetaminophen (APAP) is known to cause drug-induced liver injury (DILI) in humans. Therefore, the detection or prediction of these side-effects at an early stage using appropriate biomarkers is the need of the hour. Micro RNA (miR)-122 is expected to be a novel biomarker for liver injury. However, more evidence is required in various alternate situations such as its use in combination as APAP is often used along with anticancer drugs. In the present study, we aimed to evaluate the functions of miR-122 as a biomarker for liver injury in comparison with alanine aminotransferase (ALT) in a mice model with the APAP-induced liver injury (AILI). Consequently, there was a dose-dependent increase in miR-122 after administration of APAP intraperitoneally. Similar observations were made for ALT activity. Additionally, the expression of miR-122 increased in a more rapid manner compared to ALT activity. However, there was a variation in the miR-122 expression. Further, we investigated the drug–drug interaction between APAP and 5-fluorouracil using miR-122 and ALT in mice. As a result, the degree of AILI was not changed by the use of 5-fluorouracil in combination with APAP in mice.

Key words acetaminophen; micro RNA-122; biomarker; liver injury

Acetaminophen (APAP) belongs to the class of non-steroid anti-inflammatory drugs. Meanwhile, when high doses of APAP are administered, its metabolite, such as *N*-acetyl-*p*-benzoquinoneimine, is well known to cause hepatotoxicity.[1,2] Further, in the United States, it has been reported that overdose of APAP induced acute liver failure.[3] Alanine aminotransferase (ALT) has long been used as the gold standard for the detection of drug-induced liver injury (DILI). However, ALT was reported to be increased in conditions such as diabetes,[4] myocardial infarction,[5] or after exercise.[6] Moreover, ALT gene expression was reported to be affected by peroxisome proliferator-activated receptor α (PPARα, its agonists used as lipid-lowering drugs).[7] Therefore, the detection or prediction of the side effects of APAP at the early stages using appropriate biomarkers is urgently required.

Of all the microRNA (miR), miR-122 is the most abundantly localized in and highly specific for the liver.[8] In addition, it is reported to be rapidly released into the blood from the hepatocytes when the cells are injured and returns to normal levels soon after the cessation of treatment. Therefore, the miR-122 is considered to precisely reflect the conditions of acute liver injury (ALI).[9,10] Taking this information into consideration, miR-122 is expected to be a novel biomarker for ALI. However, more evidence is required to use this biomarker in various situations such as combination use because APAP is often used along with anticancer drugs.

In palliative care, APAP is sometimes used as an analgesic in cancer patients taking 5-fluorouracil (5-FU) as chemotherapy. Similarly, it was reported that severe chronic hepatitis was induced by Tegafur, a prodrug of 5-FU, and Uracil.[11] Therefore, it is suggested that a combination of APAP and 5-FU could increase the frequency or severity of the DILI by drug–drug interaction. However, this drug–drug interaction between APAP and 5-FU has not yet been clarified.

In this study, we aimed to evaluate the usability of miR-122 as a biomarker for ALI in the APAP-induced liver injury (AILI) mice model in comparison with ALT.[12] Further, we investigated the drug–drug interaction between APAP and 5-FU using miR-122 and ALT in mice.

MATERIALS AND METHODS

Chemicals Pharmaceutical grade APAP and 5-FU were provided by Towa Pharmaceutical Co., Ltd. (Osaka, Japan).

Cells HepG2 cells were cultured in Dulbecco's modified Eagle's medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan) containing 10% fetal bovine serum (Bovogen Biologicals Pty. Ltd., Victoria, Australia) and 1% penicillin–streptomycin-L-glutamine solution (100×, Wako Pure Chemical Industries, Ltd.) at 37°C under 5% CO_2.

Cytotoxicity HepG2 cells were seeded in 24 well plates with a density of 8.34×10^4 cells/cm^2 and cultured. After 24 h, the following drugs were added to the HepG2 cells respectively; 5-FU (10 μM), APAP (20 mM), and a combination of 5-FU (10 μM) and APAP (20 mM). After 24 h, the media were removed and the cellular viability was analyzed using Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan).

Animals C57BL/6 male mice (8 weeks old) were pur-

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chased from CLEA Japan, Inc. (Tokyo, Japan). All the animal experiments were carried out in accordance with the Guide for Animal Experimentation of Nagasaki University (approval number: 1706011381-2). These mice were fasted for 20 h before administration of APAP and/or 5-FU.

Drug Administration and Blood Sampling APAP and/or 5-FU were administrated intraperitoneally to the mice. Blood were collected *via* the tail vein immediately before and after administration. To prepare serum for ALT measurement, the blood were centrifuged at 5000×***g*** for 10 min at 25°C. To obtain plasma samples for miR-122 measurement, the blood were centrifuged at 1200×***g*** for 15 min at 4°C after adding ethylenediaminetetraacetic acid (EDTA).

Evaluation of ALT Levels in Serum The ALT activity in the serum was measured by colorimetric method as previously described.[13)]

Measurement of miR-122 Expression in Plasma The total miRs in plasma were extracted using the miRNeasy® serum/plasma kit (QIAGEN, Hilden, Germany). The extracted total miRs were applied to RT-PCR and miR-122 expression levels were measured by the ΔΔCt method.

Statistical Analysis Significant differences between the groups were determined by ANOVA and Tukey's test. Pearson's correlation coefficient was used to calculate the correlation coefficient. $p<0.05$ was considered statistically significant.

RESULTS

Evaluation of ALT and miR-122 at 3 h after an Administration of APAP ALT activity and miR-122 expression were measured at 3 h after administering 100, 200, or 300 mg/kg of APAP. In Fig. 1B, miR-122 expression reached a plateau at 200 mg/kg APAP, which was similar to the behavior shown by ALT (Fig. 1A), although miR-122 expression showed greater variation. In addition, the coefficient of correlation between ALT and miR-122 was found to be 0.89 ($p<0.001$) (Fig. 1C).

Time–Course Profiles of ALT Activity and miR-122 Expression after an Administration of APAP The ALT activity and miR-122 expression were measured at 0, 3, 6, and 24 h after administration of 300 mg/kg APAP. ALT activity increased until 6 h after administration and then gradually decreased (Fig. 2A). On the other hand, miR-122 expression reached a peak at 3 h after administration and decreased rapidly to 1 to 0.1% of the peak until 24 h (Fig. 2B). The miR-122 expression varied greatly compared to ALT activity (Fig. 2).

Evaluation of Cellular Damage by a Combination of APAP and 5-FU The cellular viability of HepG2 was evalu-

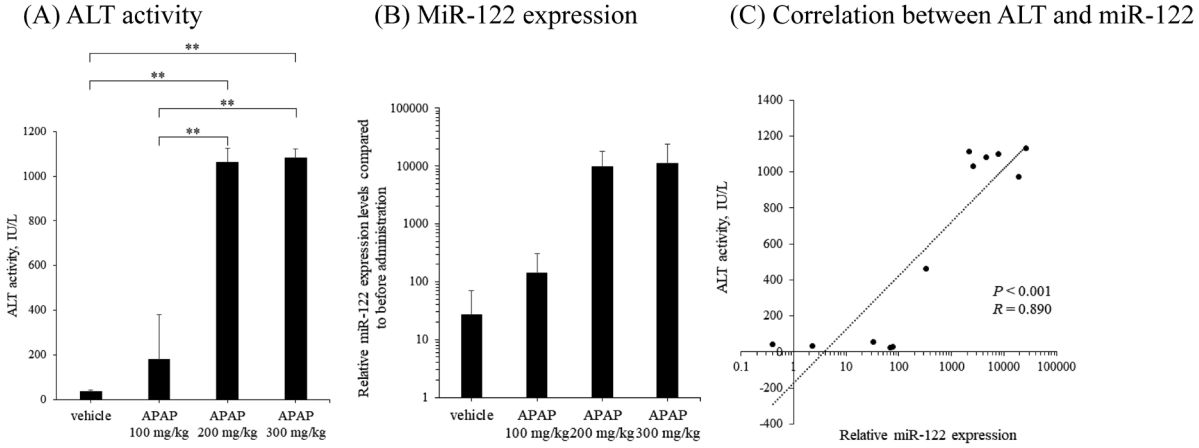

Fig. 1. Results of the ALT Activity (A) and miR-122 Expression (B) at 3 h after Administration of APAP (100, 200, or 300 mg/kg) (n=3) and the Correlation between the ALT Activity and miR-122 Expression Evaluated by Pearson's Correlation Coefficient (n=12) (C)

The data is represented as the mean±standard deviation. ** $p<0.01$, Tukey's test following ANOVA.

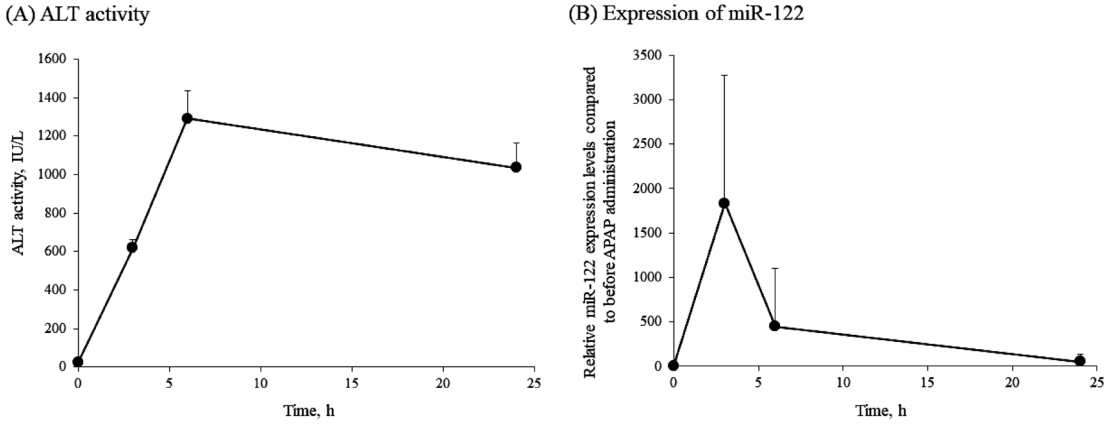

Fig. 2. Time–Course Profiles of ALT Activity (n=3) (A) and miR-122 Expression (n=3) (B) after Administration of 300 mg/kg APAP

The data is represented as the mean±standard deviation.

ated at 24 h after addition of APAP (20 mM), 5-FU (10 μM), or a combination of APAP (20 mM) and 5-FU (10 μM). In Fig. 3, the cellular viability after addition of 5-FU was similar to that of the control group. In contrast, the cellular viability after addition of APAP decreased to 55.3%. In the combination group, the cellular viability decreased to 45.1% and was significantly lower in comparison with the APAP group ($p<0.05$).

Effect of Combination Use of APAP and 5-FU on the DILI in Mice To evaluate the effect of APAP and 5-FU in combination on the DILI, the ALT activity and miR-122 expression were measured after administration of APAP, 5-FU, and APAP combined with 5-FU. Resultantly, both, the ALT activity and miR-122 expression in the combination group were not different from those in the APAP group ($p>0.05$) (Fig. 4).

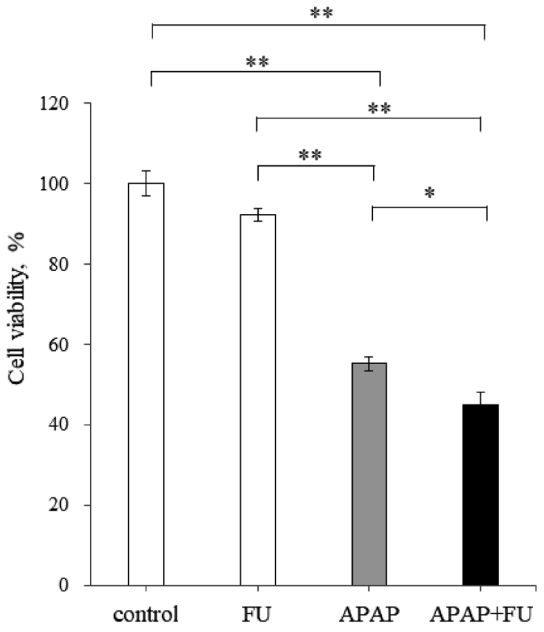

Fig. 3. Results of the Cellular Viability of HepG2 at 24 h after Administration of the Following Drugs; Control, 10 μM FU, 20 mM APAP, and 20 mM APAP Combined with 10 μM FU

The data is represented as the mean±standard deviation ($n=3$). *$p<0.05$; **$p<0.01$, Tukey's test following ANOVA.

DISCUSSION

The miR-122 is expected to be a novel biomarker for AILI, however, little has been reported about whether the miR-122 is applicable in the detection of AILI. In this study, we compared miR-122 with ALT activity, in an AILI mice model.

First, ALT activity and miR-122 expression in the mice were measured at 3 h after administration of APAP (100, 200, or 300 mg/kg). Consequently, ALT activity increased in a dose-dependent manner after administration of APAP (Fig. 1A). In addition, the expression of miR-122 was similar to that of ALT (Fig. 1B). Furthermore, the correlation coefficient between ALT and miR-122 was found to be 0.89 ($p<0.001$) (Fig. 1C). Su *et al.* reported that the both, the serum miR-122 and ALT showed a dose-dependent increase after oral administration of APAP in Sprague-Dawley rats.[14] Our findings corroborate the same. Therefore, miR-122 can detect AILI as well as ALT activity.

Secondly, the time-course profiles of ALT and miR-122 at 0, 3, 6, and 24 h after administration of 300 mg/kg APAP were evaluated to clarify the characteristics of ALT and miR-122 in detail. In Fig. 2A, ALT activity reached a peak at 6 h after administration and decreased to about 80% after 24 h. Meanwhile, expression of miR-122 increased within 3 h and decreased rapidly to about 1 to 0.1% of the peak until 24 h. Su *et al.*[14] and Park *et al.*[15] revealed that the expression of miR-122 increased in a more rapid manner compared to ALT activity in rats when APAP was administered orally, which corresponds to our results. However, large variations in the expression of miR-122 were observed (Figs. 1B, 2). These findings suggest that miR-122 reflects the conditions of ALI more sensitively than ALT, though not as precisely when used alone. Taking these findings into consideration, we can suggest that miR-122 can be used as a complementary biomarker for the detection of AILI.

In palliative care, APAP is sometimes used along with

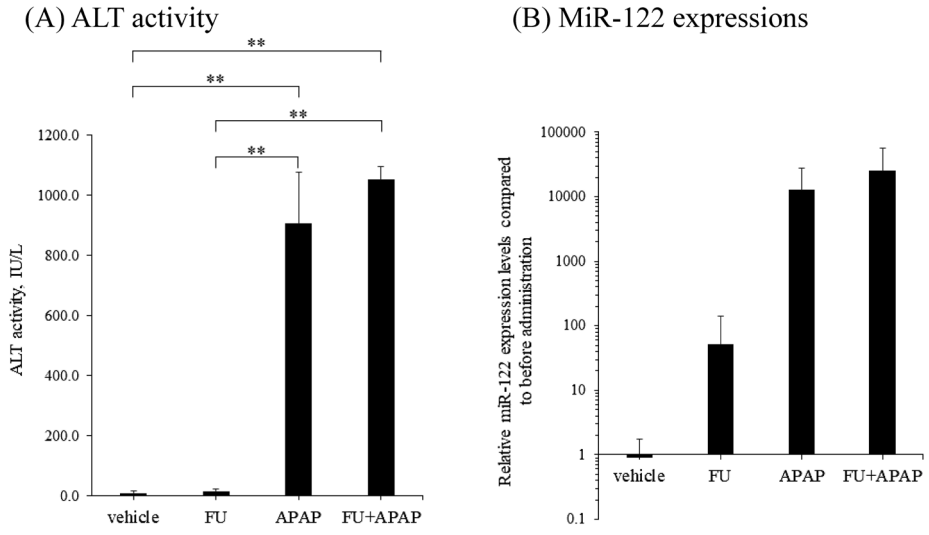

Fig. 4. ALT Activity ($n=5$) (A) and miR-122 Expressions (B) ($n=6$ to 10) at 3 h after Administration of the Following Drugs; Vehicle, 30 mg/kg FU, 300 mg/kg APAP, and 30 mg/kg FU Combined with 300 mg/kg APAP

Data is represented as the mean±standard deviation. **$p<0.01$, Tukey's test following ANOVA.

5-FU. Both the drugs are known to cause DILIs. Therefore, it is of much concern that the risk for the DILI increases by the concomitant use of APAP and 5-FU. However, the drug–drug interaction between the drugs has not yet been clarified. In the present study, the drug–drug interaction between APAP and 5-FU was investigated *in vitro* and *in vivo*. Firstly, the cellular viabilities of HepG2 were evaluated after addition of APAP, 5-FU, or a combination of both. In Fig. 3, the cellular viability of the 5-FU group was observed to be equivalent to that of the control group. On the other hand, the cellular viability decreased to 55.3% in APAP group. In addition, the viability of the combination group was significantly lower than that of the APAP group. These results may indicate that a combined use of APAP and 5-FU increases the risk of DILIs.

To assess the drug–drug interaction between APAP and 5-FU *in vivo*, the levels of ALT and miR-122 were measured after administration of the following drugs in mice; APAP, 5-FU, and a combination of both. Consequently, neither the ALT nor miR-122 in the combination group was different compared to the APAP group (Fig. 4). It is estimated that the combined use of 5-FU with APAP does not exacerbate the AILI in mice at the present dose. Further investigation is required to clarify the details of the drug–drug interaction. Meanwhile, the levels of miR-122 in 5-FU group increased at 3 h after administration although the levels of ALT were not different from that of the control group. The difference of sensitivity between miR-122 and ALT after 3 h corresponds with Fig. 2. We estimated that miR-122 could be used to detect early stages of ALI in a more rapid and sensitive manner than ALT. However, the relation between the levels of miR-122 expression and the onset or degree of the ALI is not clear. Therefore, it may be necessary to define the criterion value of miR-122 to determine the ALI in future studies.

CONCLUSION

We aimed to evaluate the function of the miR-122 as a biomarker for the detection of ALI in an AILI mice model in comparison with ALT. Our results suggest that miR-122 is applicable as a complementary biomarker to detect AILI.

Additionally, we investigated the drug–drug interaction between APAP and 5-FU using miR-122 and ALT. Although further investigation is needed to clarify the detailed drug–drug interaction, the levels of ALT and miR-122 induced by APAP were not changed when combined with 5-FU in mice, suggesting there is little interaction at these doses. To utilize miR-122 as a biomarker for the detection of ALI, further investigation of the same is necessary.

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Conflict of Interest Hatsune Enomoto and Hidehisa Tachiki are associated with Towa Pharmaceutical Co., Ltd., as employees. The other authors declare no conflict of interest.

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