Reviewer #2:

Scientific Quality: Grade B (Very good)

Language Quality: Grade B (Minor language polishing)

Conclusion: Minor revision

Specific Comments to Authors: The design of this clinical study is innovative and has certain cl inical value. This study explored the association between the early high level of serum tacrolim us and recurrence of hepatocellular carcinoma in ABO-incompatible liver transplantation. And fo und that patients with high tacrolimus concentrations at 4 weeks had significantly poor recurren ce free survival after propensity score matching. This may indicate that careful monitoring and control of tacrolimus levels are imperative in ABOi LT recipients with HCC. The results of this study may have clinical application value. However, there are some shortcomings in this study. The results of this single-center study with a small sample size may be biased. In addition, the re are many influencing factors and confounding factors in this study, and the authenticity of th e research results needs to be further verified.

1. It is suggested to further enlarge the **sample size** and **adjust for confounding factors** to i mprove the reliability of the study results.

Author's response) We totally agree with the reviewer's comment. As the reviewer pointed out, this was a single-center, small-sized study with a retrospective design. To complement this limit ation, we recruited **10 more recipients** with ABOi LT, including **3 patients** with HCC. (revised manuscript lines 145-146) As a result, propensity-score matching resulted in the selection of **12 patients** in each group. (revised manuscript line 198). Most findings were consistent before a nd after the addition of study samples, which were highlighted throughout the manuscript and r evised figures. However, the statistical significance regarding the difference in tacrolimus concen tration at 24 weeks between graft survival and loss groups was diminished, whereas survival di fference between tacrolimus high and low groups was still observed, as follows.

"The tacrolimus concentration at 24 weeks post-LT tended to be greater in the graft survival gr oup than in the graft loss group (mean 6.1 versus 4.6 ng/mL, **P = 0.088**) (Figures 2A and B). In addition, when we categorized these patients into those with tacrolimus trough concentration > 5.4 ng/mL (n = 21) and ≤ 5.4 ng/mL (n = 14), the former subgroup had better graft surviva l ($P < 0.001$) (Figure 2C)" (revised manuscript lines 282-287)

In terms of the adjustment for confounding factors, we have tried to perform additional analyse s to complement this limitation throughout the manuscript.

First, we investigated whether **cumulative tacrolimus level from 20 to 28 weeks** after LT is associated with graft survival in **non-HCC recipients**. As a result, lower cumulative tacrolimus level from week 20 to 28 ≤ 296.8 ng/mL was associated with poor graft survival (Figure S5A). We further obtained the tacrolimus level at 20-24 and 24-28 weeks after LT and calculated the mean tacrolimus level to identify its association with graft survival in non-HCC patients. As a result, the mean level of tacrolimus ≤ 5.0 ng/mL from week 20 to 28 was associated with poor graft survival in non-HCC patients (Figure S5B). (revised manuscript lines 287-294)

Second, we calculated the **cumulative level of tacrolimus**, higher cumulative tacrolimus level **from LT to week 4** > 200.2 ng/mL was also associated with a higher future **risk of HCC recurrence** (Figure S7B). In addition, we analyzed whether the mean tacrolimus trough level within 4 weeks after LT, which was obtained from week 1/2/3/4 timepoints, is associated with the HCC recurrence, and a higher mean level of tacrolimus from LT to week 4 > 8.0 ng/mL was associated with a higher risk of HCC recurrence (Figure S7C). (revised manuscript lines 330-336)

Third, we performed **subgroup analyses** according to the tumors **within Milan** criteria (n = 29), and **outside Milan** criteria (n = 18), and found that the patients with tacrolimus trough concentration > 7.3 ng/mL at 4 weeks post-LT also had poorer RFS (Figure 3D). (revised manuscript lines 341-343)

Fourth, we newly performed **subgroup analyses** according to the **vascular invasion and AFP** levels, high tacrolimus at 4 weeks was also associated with poor RFS (Figure S7E and F). (revised manuscript lines 343-345).

Although there might be substantial limitations, we have tried to perform various subgroup analyses and complementary analyses for tacrolimus levels, although they need to be validated in the future, larger studies.

2. In addition, it is suggested that the discussion part of this study **should be further discussed based on the research results, and compared with the relevant researches** at home and abroad.

Author's response) As the reviewer suggested, we have tried to further discuss based on the research results and comparing with the relevant researches via the revision of the pre-existing paragraphs. These revisions were indicated in the revised texts with yellow highlights, and followings are the examples.

"The long-term outcomes of ABOi LT have been reported in several studies. Data from a long-term Japanese registry showed a 5-year overall survival rate of 74.0%. Korean studies have reported 5-year survival rates of 74%–86%, reporting no differences between the HCC and non-H

CC groups, **which is consistent with our findings. In contrast to previous studies, we analyzed the risk factors for poor survival including AMR and HCC recurrence in ABOi LT**. In particular, previous studies did not identify the risk factors associated with AMR, although **we investigated the potential factors in detail including serial tacrolimus concentrations, PRA, T-cell or B-cell cross-matching, and HLA typing**. Furthermore, we showed that tacrolimus concentrations were differentially associated with graft outcomes in HCC and non-HCC patients.." (revised manuscript lines 359-368)

"**The key finding of this study is that early high exposure to tacrolimus can be associated with poor outcomes in HCC patients who undergo ABOi LT, as has been shown in cases of ABOc LT**. The trough concentration of tacrolimus in the maintenance phase can be adjusted according to whether or not the LT recipients have HCC. Regardless of ABO compatibility, the recommended tacrolimus concentration is 5–10 ng/mL during the first 3 months after LT and 5–8 ng/mL for the next 3–12 months, followed by a further reduction to approximately 3–6 ng/mL if tacrolimus is not combined with other immunosuppressants such as MMF or everolimus. Another guideline recommends that the tacrolimus concentration should be set at 6–10 ng/mL during the first 3 months and subsequently reduced and maintained below 5 ng/mL for 12 months after LT. However, CNIs can promote tumor growth owing to the overexpression of transforming growth factor-beta1 and therefore increase tumor recurrence in a rat model of HCC. A previous clinical study confirmed that early exposure to high concentrations of CNIs within the first month after LT was significantly associated with HCC recurrence, with a cutoff value of 10 ng/mL. Another study showed that tacrolimus levels > 8 ng/mL 20 days after ABOc LT were associated with HCC recurrence. **Our cutoff level for tacrolimus was 7.3 ng/mL at 4 weeks, although this level requires further validation**. A recent report also showed that early cumulative high tacrolimus exposure, and **not just one level at a single timepoint**, was associated with HCC recurrence, **We also confirmed this finding in the present study. However, the occurrence of AMR was not related to serum tacrolimus levels, emphasizing that minimizing CNIs is also feasible with ABOi LT, particularly in HCC patients.**" (revised manuscript lines 369-389)

"The HCC recurrence rate following LT is reportedly 8%–20% [30], whereas we identified a recurrence rate of 27.7%. This finding might have been associated with the inclusion of more patients who did not meet the Milan criteria compared to the number of such patients recruited in previous studies. Preoperative recipient and tumor factors, such as the size and number of tumors, AFP levels, and vascular invasion, are critically associated with recurrence. One study including a considerable number of ABOi LT patients revealed that pre-LT HCC treatment, higher tumor marker levels, and lymphovascular invasion were related to HCC recurrence . Our study showed that outside Milan, vascular invasion and high AFP levels are related to HCC recurrence in ABOi LT. We then adjusted for these conventional factors and subsequently showed that a high tacrolimus level at week 4 remained a significant factor for HCC recurrence, further underscoring the importance o

f minimizing CNIs. To achieve this goal, strategies other than rituximab and CNIs, includi ng intravenous immunoglobulins associated with plasmapheresis or the addition of evero limus, together with the close monitoring of IA titers, requires further validation." (revised manuscript lines 390-403)

"Although data from ABOi LT patients are lacking, a lower CNI concentration is associated with an increased risk of donor-specific antibody formation after kidney transplantation (KT) [32, 33]. **In line with this finding, low tacrolimus levels at 24 weeks as well as cumulative levels between 20 and 28 weeks were associated with poor long-term outcomes in non-HCC pa tients in our study**. In particular, one non-HCC LT recipient with a low tacrolimus level at 24 weeks post-LT died from late AMR, and two recipients lost grafts due to ACR-induced hepatic f ailure. However, one study reported no difference in the incidence of DSA when tacrolimus con centrations were controlled within a narrow range of 4–6 ng/mL in KT. **This strategy can also be applied to ABOi LT, particularly in HCC patients. Our findings suggest that consideri ng both DSA and HCC recurrence, maintaining a narrow range of tacrolimus concentratio ns, such as between 5.4 and 7.3 ng/mL, may be helpful in ABOi LT. However, the optima l cutoff must be validated in larger prospective studies**." (revised manuscript lines 411-423)