Peer Review File

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Reviewer A

The manuscript by Zou et al. compares the carcinogenic ability of HPV16 E6/E7 and HPV58 E6/E7. They explore this by overexpressing the E6 and E7 oncoproteins from each HPV strain in 293T and U2OS cells and assess the impact on proliferation, cell cycle, apoptosis, invasion as well as in a zebrafish model. The authors provide preliminary evidence to suggest a mechanism for why HPV 16 may have stronger carcinogenic capacity and I believe that their work is worthy of publication in the journal of Translational Cancer Research provided they address the following comments:

Minor Comments

1. The manuscript would benefit from proofreading for spelling and grammatical errors.
   Reply: Thank you for your advices. And we have corrected the errors.
   Changes in the text: we have modified our text as advised
2. Throughout the manuscript “respectively” is used inappropriately and should be removed and “u” is used instead of “μ” and this should be corrected.
   Reply: Thank you for your advices. And we have corrected the errors.
   Changes in the text: we have removed the “respectively” in line 36, 81, 208, 267,398, 400, 405, 410, 411, 417, 423. And we change the “ug” to “μg” in line 115, 125 and 142. Also we change the “uM” to “μM” in line 146-147, 183, 230-231, 232, 241, 244, 293, 419-420, 423 and 427.
3. The authors should provide an explanation early in the manuscript as to why they used the 293T and U2OS cells and not “normal” keratinocytes which are the cells of origin of cervical cancer.
   Reply: Thank you for your advices. And we have added the explanation in the advised manuscript.
   Changes in the text: we have modified our text as advised (see Page 5, line 117-119)

4. Line 30: Human papilomavirus should be Human papillomavirus.

   Reply: Thank you for your advices. And we have corrected the errors.

   Changes in the text: we have modified our text as advised (see Page 2, line 30)

5. Line 45: “phosphate” should be “phosphorylated”.

   Reply: Thank you for your advices. And we have corrected the error.

   Changes in the text: we have modified our text as advised (see Page 2, line 45)

6. Line 54-55: The authors should use the updated cervical cancer global statistics.

   Reply: Thank you for your advices. And we have modified our text as advised.

   Changes in the text: we have modified our text as advised (see Page 3, line 54-55)

7. Line 56-57: HPV16 being responsible for ~75% of cervical cancer cases is incorrect.

   Reply: Thank you for your advices. And we have corrected the error.

   Changes in the text: we have modified our text as advised (see Page 3, line 60-61)

8. Line 56: “known” should be corrected to “know”.

   Reply: Thank you for your advices. And we have corrected the error.

   Changes in the text: we have modified our text as advised (see Page 3, line 56)

9. Line 58: “Asia” should be corrected to “Asian”.

   Reply: Thank you for your advices. And we have corrected the error.

   Changes in the text: we have modified our text as advised (see Page 3, line 58)

10. Line 63: write out CIN in full when first used.

    Reply: Thank you for your advices. And we have modified our text as advised.

    Changes in the text: we have modified our text as advised (see Page 3, line 63)

11. Line 68: referring to HPV16 and 58 as two viruses should be strains or subtypes of the same virus.

    Reply: Thank you for your advices. And we have modified our text as advised.

    Changes in the text: we have modified our text as advised (see Page 4, line 68)

12. Line 91: Was there a reason for the selection of the age group 25-65?

    Reply: Thank you for your question. The age of the patients included in the study was 25-65 years old.

13. Line 102-103: LSIL/HSIL should be written in full when first used.

    Reply: Thank you for your advices. And we have modified our text as advised.

    Changes in the text: we have modified our text as advised (see Page 5, line 102-103)

14. Line 208: “respectively” should be removed.

Reply: Thank you for your advices. And we have modified our text as advised.

Changes in the text: we have modified our text as advised (see Page 9, line 208)

15. Line 226-227: The authors should provide a rationale for focusing on E7.

Reply: Thank you for your advices. And we have modified our text as advised.

Changes in the text: we have modified our text as advised (see Page 12, line 288-296)

16. Line 229: “phosphorylation” should be corrected to “phosphorylated”.

Reply: Thank you for your advices. And we have corrected the errors.

Changes in the text: we have modified our text as advised (see Page 10, line 229)

17. Line 277-278: This sentence does not make sense.

Reply: Thank you for your advices. And we have modified our text as advised.

Changes in the text: we have deleted the sentence (see line 277-278).

18. Figure 1B: U2OS is missing from the data for experiments performed in them.

Reply: Thank you for your advices. And we have modified the Figure 1B as advised.

19. The western blotting data for Figure 3 A and B should quantified as shown for Figure 3C.

Reply: Thank you for your advices. And we have modified the Figure 3 A and B as advised.

Major Comments

• Figures 1 and 2: Western blotting showing the levels of transfected HPV16/58 E6 and E7 should be provided.

Reply: Thank you for your advices. And we have modified our text as advised.

Changes in the figure: we have added the supplement Figure S1 in the text. First, we constructed the plasmids contain HPV16/58 E6 and E7 into the pFLAG-CMV5.1 vector, then transfect the plasmids to 293T and U2OS cell lines. The expression of HPV16/58 E6 and E7 was measures by RT-PCR (supplement Figure S1 B). Since lacking the antibody of HPV58 E7 on the market, we used the FLAG antibody to detect the expression of HPV16/58 E6 and E7 instead. And we detect expression of HPV16/58 E6 and E7 by using the FLAG many times and the results was same as the ones in the Figure 3.

• Lines 241-246: Can the authors quantify the reduction in cell activity in their zebrafish studies?

Reply: Thank you for your advices. And we have modified our text as advised.

Changes in the text: we have added the relative fluorescence intensity of cells in Figure 4 which was measured by Image J software.

• Lines 285-289: The authors should propose future experiments that they may perform to address the relatively low levels of FLAG-E7.

Reply: Thank you for your advices. And we have modified our text as advised.

Changes in the text: we have modified our text as advised (see Page 13, line 311-314). The expression of HPV16-E7 was low in both 293T and U2OS cell lines. It may be showed that HPV16-E7 decreased the expression of RB and then disappeared, while HPV58 E7 made Rb hyper-phosphate and it can be restored by roscovitine. Further research is needed to try the assays in different time point to find out what was the different functions between HPV16 E7 and HPV58 E7 on RB.

Reviewer B

This clinical and preclinical study examines the incidence and potential oncogenic differences between HPV 16 and 58. The authors identify a potential difference in Rb deactivation that may explain differences.

1. Please include IRB information, if that is different from the Hospital Ethics Committee.

   Reply: Thank you for your advices. And we have modified our text as advised.

   Changes in the text: we have modified our text as advised (see Page 13, line 320)
2. What does "incorporate" mean in the exclusion criteria?

   Reply: Thank you for your advices. And we have modified our text as advised.

   Changes in the text: we have modified our text as advised (see Page 5, line 97-98)
3. I see that the authors explained their rationale for using these two cell lines in the discussion, but I was wondering if there are no cervical cell lines that also meet those requirements. Given the origins of osteosarcoma and kidney, I am unsure if this will be recapitulated in cervical epithelial cells.

   Reply: Thank you for your question. we thought carefully for a long time which cell lines should be chosen. Since the purpose of the study was to compare the carcinogenic ability between HPV16 and 58 E6 and E7 which mainly regulate the expression of P53 and RB. The suitable cell lines which may not contain HPV16 or 58, and also with the wild type of P53 and RB. Since the common cell lines such as SiHa (HPV16 positive), CasKi (HPV16 positive), Hela (HPV18 positive) and C33A (mutant P53) are not suitable. And normal epithelial cells

could not be passed down for cultivation in vitro and in zebrafish model. Finally, we find the suitable cell lines, 293T and U2OS, with wild type of P53 and RB and without HPV infection, which can be proliferated by passage culture to measure the difference in the biological behavior.

4. Can the authors explain what the zebrafish model adds to this manuscript? Given the injection in the intestine of cell lines that are not epithelial, I am confused what it adds.

Reply: Thank you for your question. Since we could not choose the cervical cancer cell lines in the study, 293T and U2OS could not be used in the mice model to simulate the growth of cervical cancer. The zebrafish model had the advantage of short growth cycle, transparent and visible body. The cellular activity of 293T and U2OS contain HPV16 and HPV58 E7 genes can be detected by fluorescence microscope. First, U2OS and 293T cells was transfected with the plasmids of HPV16 and HPV58 E7 genes. 48 hours later, the transfected cells was inlabeled with Dil (Solarbio, D8700) which was the common cell membrane red fluorescence probe. We collect 2*10^6 cells of each group in U2OS and 293T cells, using a needle to inject to the intestine region of zebrafish with 48h postfertilization. Then the zebrafishes with different cells transfected with HPV16 and HPV58 E7 genes were fed in water treated with DMSO or 30 μM Roscovitine for 24h. Then, Detect the fluorescence intensity of abdominal cells in each group using a fluorescence microscope under the same parameter. The fluorescence intensity indicated the cell activity of U2OS and 293T cells which was transfected with the plasmids of HPV16 and HPV58 E7 genes in the abdomen of zebrafish.