Peer Review File

Article information: https://dx.doi.org/10.21037/gs-23-127

Reviewer A

Comment 1: The abstract is not sufficient and needs further modification. The research background did not indicate the clinical needs of the research focus.
Reply 1: Thanks for your warm works. The research background has been rewritten.
Changes in the text: Salivary adenoid cystic carcinoma (SACC) is a unique malignant tumor of the salivary gland with poor prognosis，which is not effective with chemotherapy and targeted drugs. Therefore，it is important to explore the molecular mechanism underlying SACC invasion and metastasis to develop novel therapeutic strategies and targets in clinical research.(page2;line 41-45)

Comment 2: All figures are not clear enough. It is recommended to provide clearer figures again.
Reply 2: We are very sorry for the clarity of pictures and clearer figures have been uploaded respectively
Changes in the text: Inapplicability

Comment 3: What is the role of DNA methylation in solid tumor resistance? How to analyze the gene expression characteristics of methylated region-specific DNA to predict the outcome of anti-cancer treatment? It is recommended to add relevant contents.
Reply 3: We have modified our text as advised and it has been added in Introduction.
Changes in the text: The aberrantly modified DNA methylation confers unique features on tumor cells, including sustained proliferative potential, resistance to growth-suppressive or cell death signals, augmented replicative immortality, invasion, and metastasis. As a result, DNA methylation abnormalities exhibit significant impacts on all stages of oncogenesis from its onset to progression to metastasis, which may eventually lead to tumor chemotherapy resistance. Thus, DNA methylation/demethylation pathway as a promising target for therapeutic intervention in cancer (6) (page4; line 98-105)

Comment 4: It is recommended to add the expression and significance of the Wnt/β-catenin signaling pathway in salivary adenoid cystic carcinoma in the discussion.
Reply 4: We have modified our text as advised and it has been added in Discussion.
Changes in the text: Wnt/β-catenin signaling pathway has been found abnormal activation in many solid cancers，including SACC. Ji et al found CLDN7 inhibits cell proliferation and metastasis by inactivating the Wnt/β-catenin signaling in SACC(31). Fatty acid synthase was

conformed contributes to epithelial-mesenchymal transition and invasion of salivary adenoid cystic carcinoma through PRRX1/Wnt/β-catenin pathway(32). (page 18; line 540-545)

Comment 5: There are many genes that regulate tumor proliferation and metastasis in salivary adenoid cystic carcinoma. Why did the author choose AJAP1 for research? Please describe the reason.

Reply5: The GO enrichment analysis results indicated that the enrichment degree of differentially expressed genes was the highest in biological adhesion ($P<0.01$) and was mainly in the extracellular matrix organization of pathway enrichment analysis ($P<0.01$). As both of the different expressed genes in Biological adhesion and Extracellular matrix organization processes contain AJAP1, we selected AJAP1 as the candidate research target. Then, we detected the expression of AJAP1 protein in the cancer tissues and paracancerous tissues, and found AJAP1 protein was significantly higher in paracancerous tissues than cancer tissues. The results of chi-squared test showed that the downregulation of AJAP1 expression was closely related to age, lymph node metastasis/distant metastasis, and Tumor Nodes Metastasis (TNM) stage.Cox regression analysis further revealed that low AJAP1 expression was an independent risk factor for SACC prognosis. Thus, we choose AJAP1 for research.

Changes in the text: It is described in detail in the results section"##AJAP1 expression is significantly down-regulated in SACC".(page 11;line 308-326)"

Comment 6: The introduction part of this paper is not comprehensive enough, and the similar papers have not been cited, such as "Reconstruction and analysis of the aberrant lncRNA-miRNA-mRNA network based on competitive endogenous RNA in adenoid cystic carcinoma of the salivary gland, Transl Cancer Res, PMID: 35116364". It is recommended to quote this article.

Reply 6: We have modified our text as advised and it has been quoted in the article.

Changes in the text: In recent years, studies have shown that the abnormal expression of some genes may play an important role in the malignant progression of SACC.(page 4;line95-96)

5.Tang YF, Wu WJ, Zhang JY, et al. Reconstruction and analysis of the aberrant lncRNA-miRNA-mRNA network based on competitive endogenous RNA in adenoid cystic carcinoma of the salivary gland. Transl Cancer Res 2021;10:5133-5149.(page21, line 620- 623)

Comment 7: Does the signaling in this study affect the radioresistance of salivary adenoid cystic carcinoma? What impact might it have? It is recommended to add relevant contents.

Reply 7: We have modified our text as advised and it has been added in Discussion.

Changes in the text: The abnormal activation of Wnt/beta-catenin signaling pathway was found be associated with radioresistance of cervical cancer and esophageal squamous cell carcinoma, while it's role in salivary adenoid cystic carcinoma is still unclear and more research is needed.(page 18;line 546-549)

Reviewer B

It is an interesting and novel study about role of AJAP1 protein expression in adenoid cystic carcinoma. This study highlights the role of AJAP1/Ecadherin/B catenin pathway in the pathogenesis of SACC where loss of protein by promotor hypermethylation results in increase invasion through extracellular matrix.

Comment 1: In methods, for immunohistochemistry mention loss of cytoplasmic expression is positive for mutation as well as describe its expression in normal tissues such as normal salivary gland parenchyma, endothelial cells and inflammatory cells.

Reply 1: Thanks for your warm works and in our study, we found that abnormal hypermethylation of the AJAP1 promoter in SACC tumors is an important cause of the loss of AJAP1 expression.Furthermore, to verify the expression of AJAP1 in SACC tissues, we detected the expression of AJAP1 protein in the cancer tissues of patients with SACC was 44.76% (47/105), while in paracancerous tissues, the positive rate of the AJAP1 protein was 86.67% (91/105), which was significantly higher than that in cancer tissues ($P<0.01$) (Figure 1E-1I).

Changes in the text: Inapplicability

Comment 2: Mention the product size in agrose gel electrophoresis.
Reply 2: Thanks for your warm works and the product size in agrose gel electrophoresis have been added.
Changes in the text:
(F) Western blot results showed that the expression level of the AJAP1 protein(45kDa) in paracancerous tumor tissues was significantly higher than that in tumor tissues; (page24, line 723)
(C) The level of AJAP1 protein(45kDa) was increased after the introduction of overexpressed lentivirus into SACC-LM cells. (D) The expression of AJAP1 protein(45kDa) was significantly down-regulated in SACC-83 cells after the introduction of knockdown lentivirus.(page30, line 782)
(A) Immunofluorescence displayed the co-localization among AJAP1(45kDa), E-cadherin(97kDa), and β-catenin(85kDa) (×200).(page32, line 807)
(H-J) Overexpression/knockdown of AJAP1 significantly up-regulated/down-regulated the expression of β-catenin downstream genes, respectively(AJAP1, 45kDa; C-myc, 49kDa; CyclinD1, 33kDa; MMP1, 54kDa; GAPDH, 36kDa). *$P<0.05$. (page33, line 815-816)

Comment3-a: B catenin which is chiefly mutated in basal cell adenoma and adenocarcinoma of salivary gland, and have been used as diagnostic marker to differentiate from its chief differential i.e. adenoid cystic carcinoma. Your study highlights how with higher grade of adenoid cystic carcinoma with loss of AJAP1 it can also express nuclear B catenin. Thus, one need to rely on MYB FISH for exact diagnosis.
Reply 3-a: Thanks for your warm works, there is no doubt that MYB FISH tests are more persuasive. But in our study, we have detected the expression of nuclear beta-catenin by Western Blot and the result showed AJAP1 reduces the nuclear localization of β-catenin by forming the AJAP1/E-cadherin/β-catenin complex.
Changes in the text: Inapplicability

Comment3-b: Do authors recommend evaluation of AJAP1 by IHC in all cases of adenoid cystic carcinoma to determine its prognosis.

Reply 3-b: Thanks for your warm works and we have detected the expression of AJAP1 by IHC in all cases of adenoid cystic carcinoma, and found that low AJAP1 expression was an independent risk factor for SACC prognosis. (page12,339-345)
Changes in the text: Inapplicability

Reviewer C

1. Your abstract is too long. The abstract should be 200-350 words, but you have 368. Please modify.

Reply 2: The abstract has been modified within 350 words.

Changes in the text: Background: Salivary adenoid cystic carcinoma (SACC) is a unique malignant tumor of the salivary gland with poor prognosis, which is not effective with chemotherapy and targeted drugs. Therefore, it is important to explore the molecular mechanism underlying SACC invasion and metastasis to develop novel therapeutic strategies and targets in clinical research.

Methods: Real-time Quantitative Polymerase Chain Reaction (RT-qPCR) and western blot were performed to detect the expression of Adherens Junctions Associated Protein 1 (AJAP1). Methylation-specific PCR was used to evaluate the methylation of the AJAP1 promoter. AJAP1 was overexpressed or knocked down by lentivirus-mediated transfection. Kaplan-Meier analysis was conducted to create a survival curve and the log-rank test was used to analyze the overall survival (OS). The prognostic correlation was assessed using univariate and multivariate Cox regression analyses. Co-Immunoprecipitation (Co-IP) was utilized to pull down the possible binding protein of AJAP1 and laser scanning confocal microscopy was applied to detect the subcellular localization of AJAP1, E-cadherin, and β-catenin. Cell viability, colony formation, wound healing, and Transwell invasion assays were performed to evaluate the function of AJAP1 in vitro. A subcutaneous xenograft assay in nude mice was performed to verify the function of AJAP1 in vivo.

Results: AJAP1 was downregulated in SACC tumors and was closely related to SACC lymph node/distant metastasis, which was an independent risk factor for SACC prognosis. Methylation-specific PCR confirmed that high methylation of the AJAP1 promoter was the main cause of its silencing. Overexpression or knockdown of AJAP1 in SACC cells could significantly inhibit or promote the proliferation, invasion, and metastasis of SACC cells, respectively, in both the in vitro and in vivo experiments. Mechanically, we found that AJAP1 binds to E-cadherin and β-catenin to form a complex in cytomembrane, reducing the nuclear translocation of β-catenin and blocking the Wingless / Integrated /β-catenin(Wnt/β-catenin) signaling pathway to play a suppressive role in cancer.

Conclusions: In conclusion, these results suggest that the downregulation of AJAP1 protein expression may play a certain role in progression and metastasis of SACC. Our study indicates that AJAP1 may be a potential prognostic molecular marker and therapeutic target for SACC.(page2-3;line 43-75)

2. Please check if any more references need to be added in the below 3 sentences since you mentioned “Studies”, but only one reference was cited. If not, “studies” should be changed to “a study/a previous study”.

98 In recent years, studies have shown that the abnormal expression of some genes
99 may play an important role in the malignant progression of SACC (5) and
100 methylation of DNA promoter may be one of the main causes of abnormal gene
101 expression. The aberrantly modified DNA methylation confers unique features on

498 negatively regulated by miR-552. Other studies have shown that a high degree of
499 methylation exists in the 1p36 region in some tumors, which may explain the loss of
500 expression of related tumor suppressor genes (24). Through database retrieval and

536 bind directly to E-cadherin (29). Indeed, direct binding between E-cadherin and
537 β-catenin has been found in several studies (30). Therefore, in this study, the

Reply 3: We have modified our text as advised.

Changes in the text:

In recent years, a previous study have shown that the abnormal expression of some genes may play an important role in the malignant progression of SACC (5) (page4;line 97-98)

A previous study has shown that a high degree of methylation exists in the 1p36 region in some tumors, which may explain the loss of expression of related tumor suppressor genes (24).(page17 ;line493-495)

Indeed, direct binding between E-cadherin and β-catenin has been found in a previous study(30). (page18 ;line 531-532)

3. Table 2-3:

Please indicate the full name of “TMN” in Table 2 footnote and “OS”, “SACC” “TMN” in Table 3 footnote.

Reply 4: We have indicated the full name of “TMN” in Table 2 footnote and “OS”, “SACC” “TMN” in Table 3 footnote and uploaded the new tables.

Changes in the text:

Table2:Footnote: TNM stage: Tumor， Lymph Node， Metastasis stage; *p<0.05(page 37;line868)

Table3:Footnote: OS:Overall Survival; TNM stage: Tumor，Lymph Node，Metastasis stage; SACC: Salivary adenoid cystic carcinoma(page 38;line 872)

4. Figure 1:

Please indicate the full name of “GO”, “IHC” in the legend.

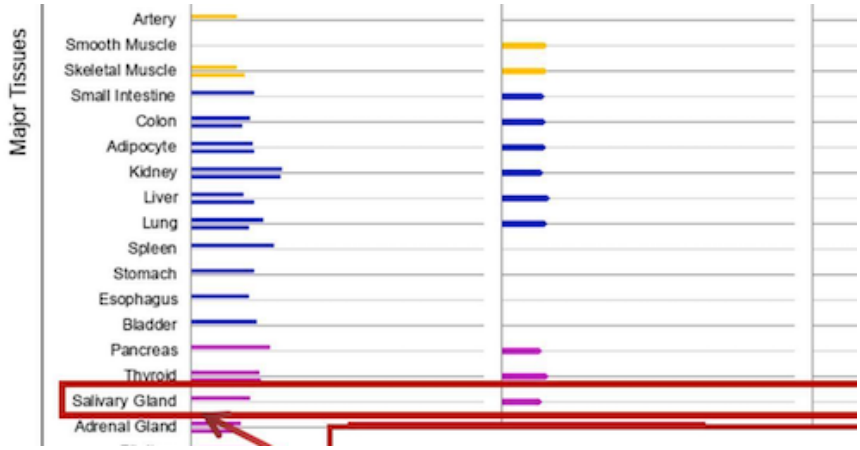
Reply 5: We have indicated the full name of “GO”, “IHC” in the legend.

Changes in the text:

Footnote：GO:Gene Ontology; IHC: Immunohistochemistry(page 25;line731)

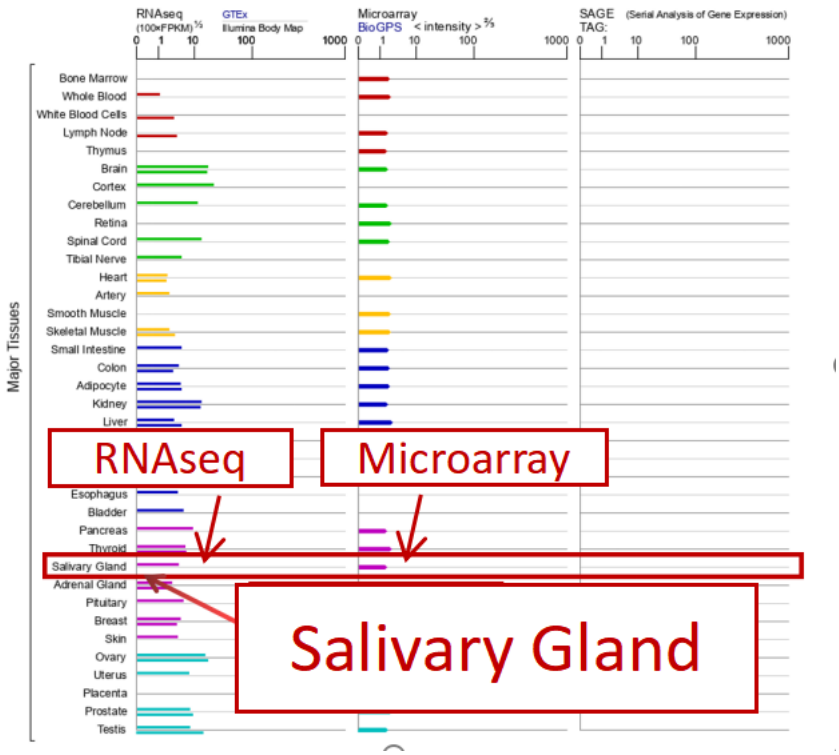
5. Figure 3:

1) Please check whether it's needed to indicate the colorful bars in Figure 3A or Figure 3A legend.

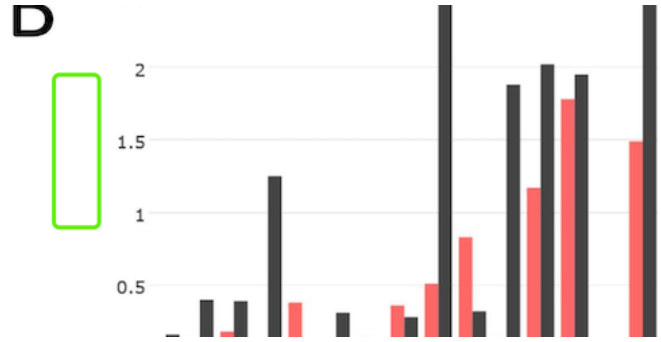


Reply 1): We have revised in FIG3.

Changes in the text:

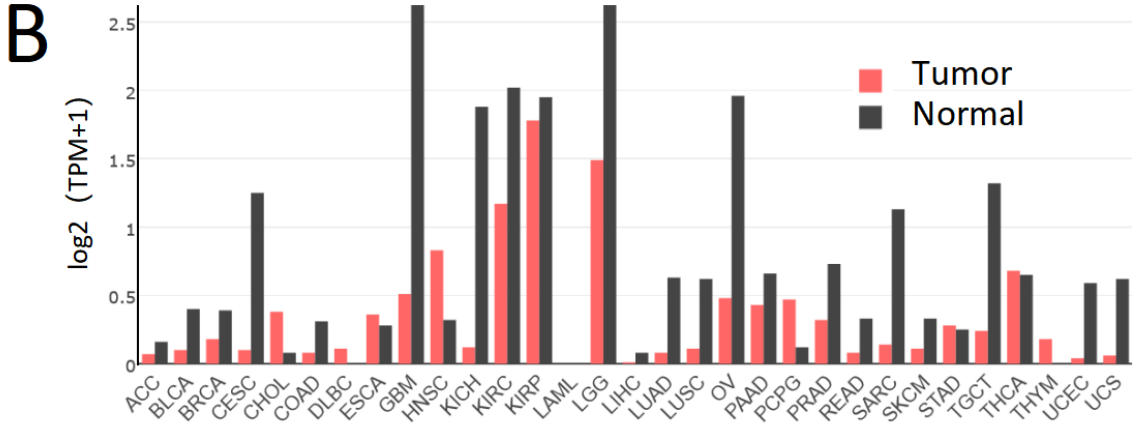


2) Please add the description of the y-axis in Figure 3B.

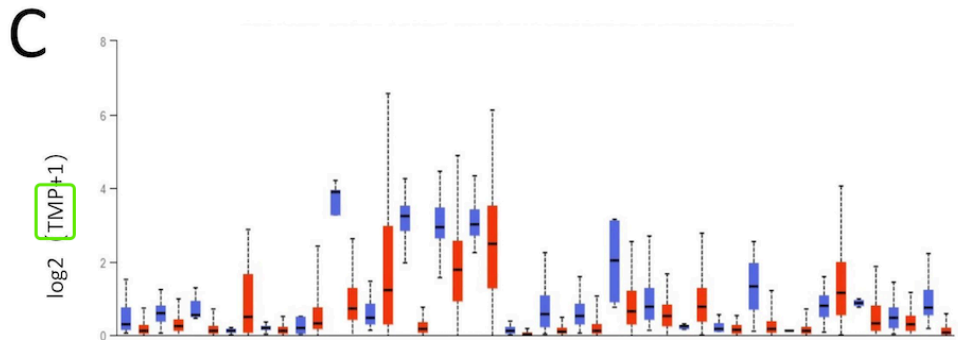


Reply 2): We have revised in FIG3.

Changes in the text:

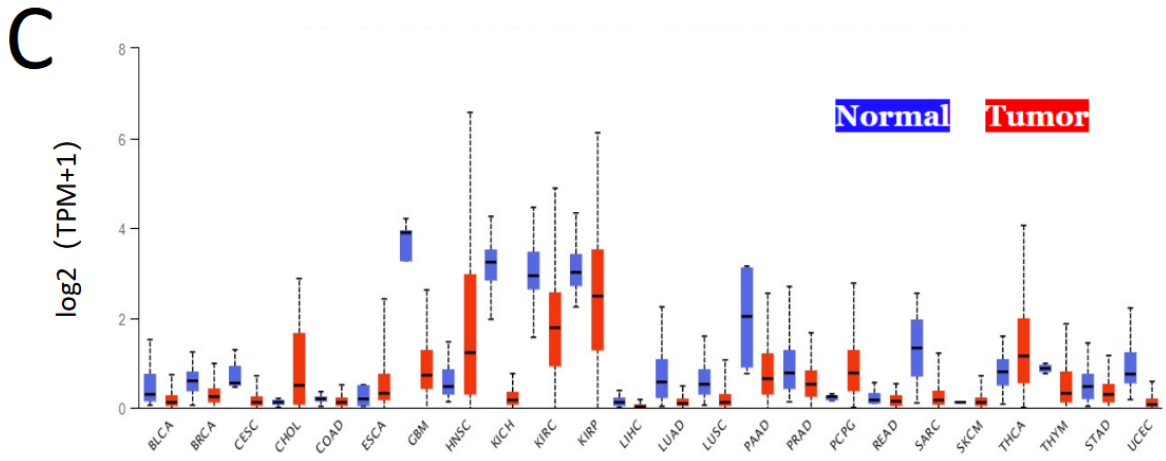


3) Figure 3C: the below word should be "TPM"? And please add labels to indicate the meaning of red and blue bar in Figure 3C.

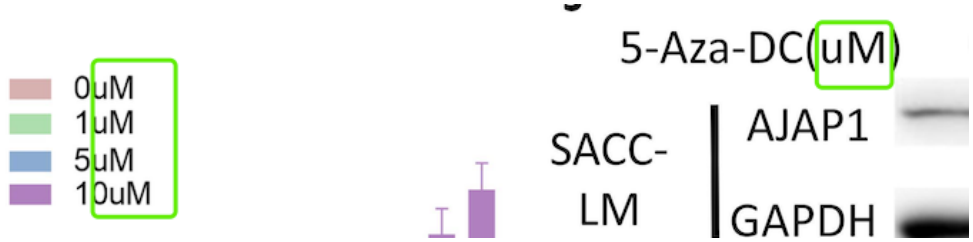


Reply 3): We have revised in FIG3.

Changes in the text:



4) Should the unit "uM" be "μM"?



Reply 4): We have revised in FIG3.

Changes in the text:

6. Figure 4:

1) Please indicate the meaning of ** in the legend.

Reply 1): We have indicated indicate the meaning of ** in the legend.

Changes in the text: Note: AJAP1, adherens junctions associated protein 1; SACC, salivary adenoid cystic carcinoma;**P<0.01;*P<0.05(page31 ;line802-803)

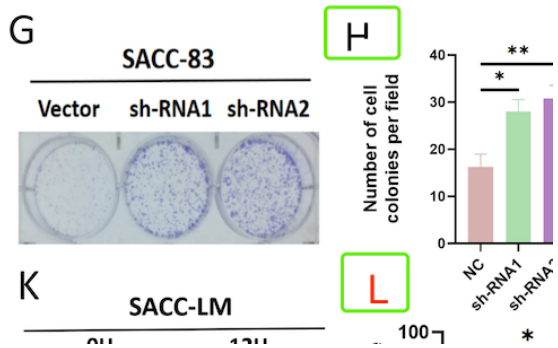
2)Please indicate the staining method and observation method for Figure 4K and M in the legend.

Reply 2): We have revised in FIG4.

Changes in the text: (K,L) The scratch healing assay showed that the migration ability was significantly down-regulated after overexpression of AJAP1 in SACC-LM cells (white light ,

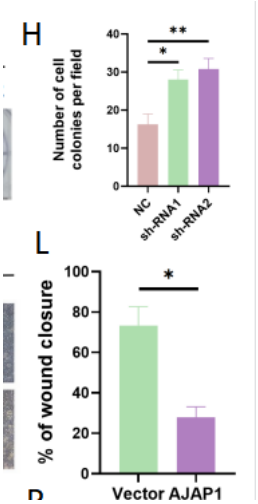
high contrast resolution , ×100). (M,N) The scratch healing assay showed that the migration ability was significantly up-regulated after AJAP1 knockdown in SSAC-83 cells (white light , high contrast resolution , ×100).(page 31;line792-797)

3) The "H" part is not clear and "L" part is missing in the figure. Please revise.



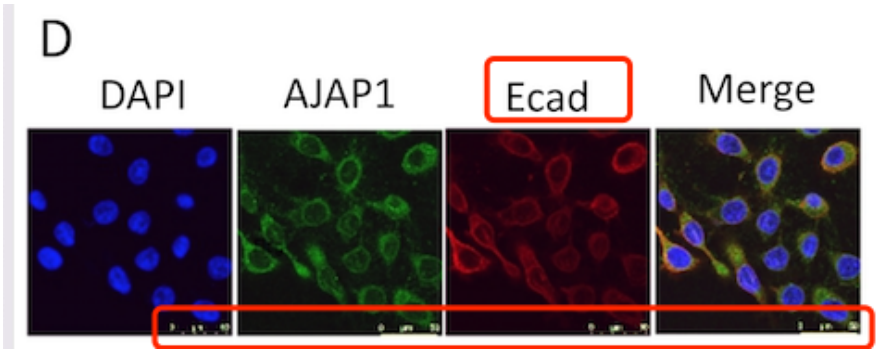
Reply 3): We have revised in FIG4.

Changes in the text:



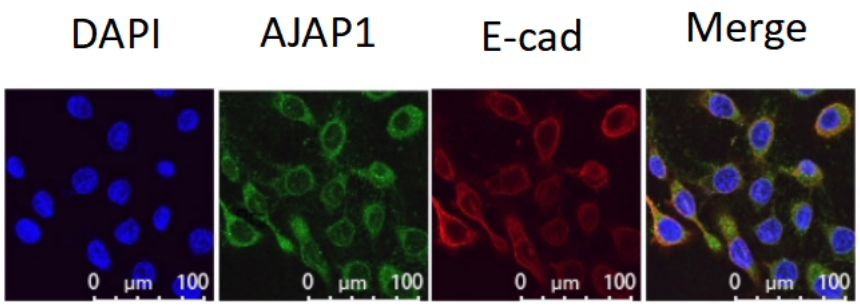
7. Figure 5:

1) The scale bars are not clear in Figure 5D. Please provide clearer Figure 5 to us. And please revise "Ecad" to "E-cad".



Reply 1): We have revised in FIG5.

Changes in the text:



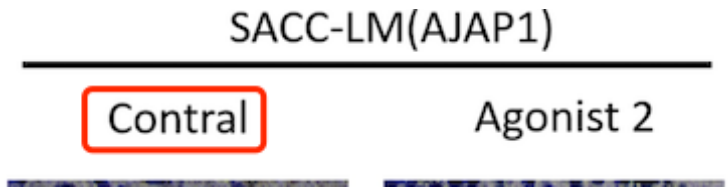
2) Please indicate the full name of "DAPI", "NC" in the legend.

Reply 2): We have revised in FIG5 legend.

Changes in the text: Note: AJAP1, adherens junctions associated protein 1; SACC, salivary adenoid cystic carcinoma; co-IP: Co-Immunoprecipitation; DAPI:4',6-diamidino-2-phenylindole; NC: normal control.(page33 ;line819-821)

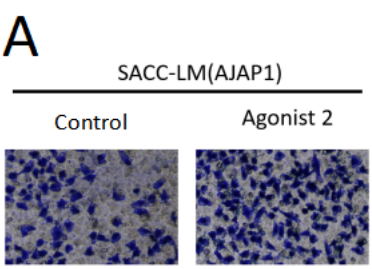
8. Figure 6:

1) Please check whether the word should be "Control"?



Reply 1): We have revised in FIG6 .

Changes in the text:



2)Please indicate the staining method and observation method for Figure 6E and G in the legend.

Reply 2): We have revised in FIG6 legend.

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Changes in the text: (E,F) Wnt/β-catenin agonist 2 could reverse the migration ability downregulation of SACC-LM cells induced by AJAP1 overexpression(white light， high contrast resolution，×100); (G,H) LF3 could reverse the migration ability upregulation of SACC-83 cells induced by AJAP1 knockdown(white light， high contrast resolution，×100);(page33 ;line819-821)

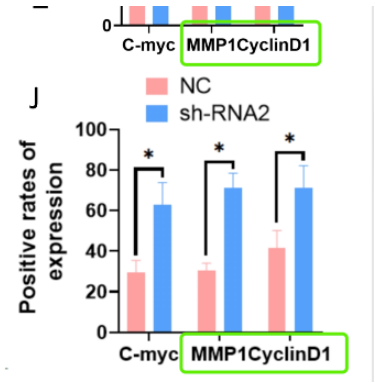
3) Please indicate the full name of "OD" in the legend.

Reply3): We have revised in FIG5 legend.

Changes in the text: Note: AJAP1, adherens junctions associated protein 1; SACC, salivary adenoid cystic carcinoma; Wnt/β-catenin signaling pathway: Wingless / Integrated /β-catenin signaling pathway; OD 450: Optical Density 450(page34 ;line830-834)

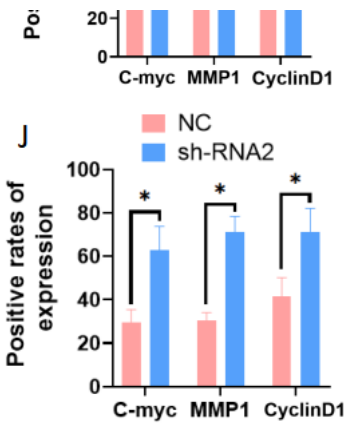
9. Figure 7:

1) The two words are too close. Please add space between them.



Reply1): We have revised in FIG7.

Changes in the text:



2) Please indicate the full name of "NC" in the legend.

Reply2): We have revised in FIG7 legend..

Changes in the text: Note:AJAP1:adherens junctions associated protein 1; SACC: salivary adenoid cystic carcinoma;NC: normal control.(page36 ;line853-854)