Peer Review File

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Reviewer A

Comment (1): The primary focus of this paper pertains to the role of long non-coding RNA (lncRNA) in adrenocortical carcinoma (ACC). Existing literature and reviews on lncRNA and miRNA in ACC have been documented (PMID: 35883677). It is imperative to incorporate citations and elucidate upon the known aspects of lncRNA in ACC within the introduction or discussion section.

Reply (1): Thank you very much for the valuable comments. Incorporating the findings from PMID: 35883677, we enhance the introduction of our text by including additional insights on the role of long non-coding RNAs (lncRNAs) and microRNAs (miRNAs) in the development of adrenocortical cancer (ACC), thus strengthening the depth of our narrative.

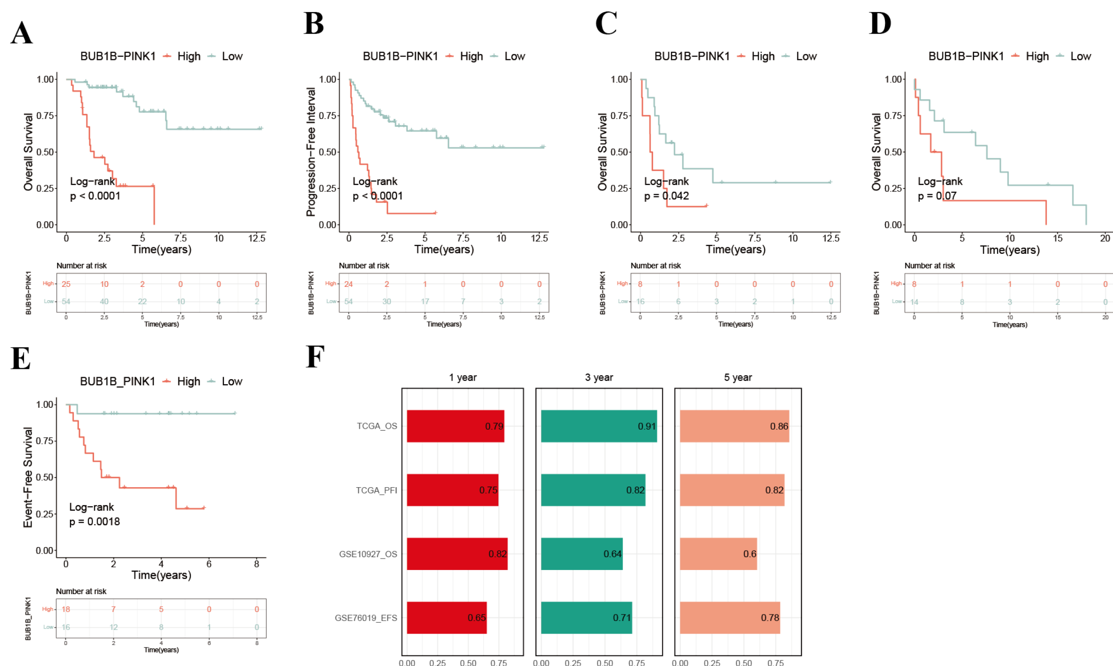
A large number of studies have shown that, in addition to BUB1B-PINK1, IGF2, and GOS2, a significant number of miRNAs and lncRNAs are also utilized in the diagnosis and prognosis prediction of ACC. In ACC, miR-100 can activate the MAPK signaling pathway through CXCR7 to induce tumor progression, and some patients currently benefit from CXCR7-targeted therapy. In vitro studies have indicated that miR-375 plays a role in the pathogenesis of ACC by regulating the PI3K/Akt pathway. Circ-CCAC1 overexpressed in ACC can enhance ACC cell proliferation, migration, and invasion by modulating miR-514a-5p, and is linked to unfavorable patient prognosis.

Changes in the text: (see Page 4, line 72-78.)

Comment (2): Molecular markers that predict the prognosis of ACC, such as the expression of BUB1B-PINK1, IGF2, and methylation status of G0S2, are well-established. Reference to these molecular markers is requisite. It is possible that there are markers, amenable to comparative analysis utilizing the databases used by the authors. Furthermore, a comparative discussion on the prognostic utility of ZFHX4-AS1 in relation to these molecular markers is warranted.

Reply (2): Thank you very much for the valuable comments. Thank you for pointing out that

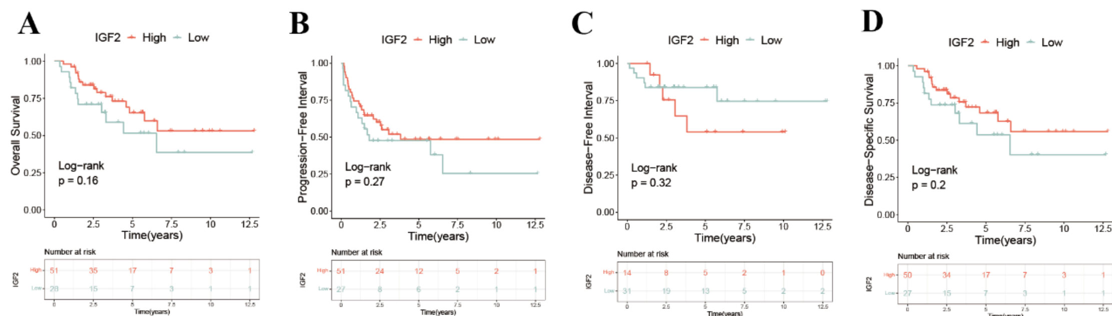
we indeed lacked a description of current landmarks in the narrative of the article. We have included this section of the narrative in the introduction of the article. (see Page 4, line 62-66.) Simultaneously, to assess the prognostic predictive efficacy of ZFHX4-AS1 alongside these markers, we conducted a comparative analysis. Initially, we established cutoff values across the four datasets to segregate samples into high and low expression cohorts. Subsequently, survival analysis was conducted, followed by receiver operating characteristic (ROC) analysis to evaluate the predictive performance, with the area under the curve (AUC) serving as the metric for utility assessment.



Result1 Survival analysis and performance evaluation of BUB1B-PINK1. (A,C,D) Kaplan-Meier survival analysis between the high and low BUB1B-PINK1 groups across 3 OS cohorts. (B) Kaplan-Meier survival analysis between the high and low BUB1B-PINK1 groups in TCGA-ACC (PFI). (E) Kaplan-Meier survival analysis between the high and low BUB1B-PINK1 groups in GSE76019 (EFS). (F) Time-dependent ROC analysis for predicting OS/PFI/EFS at 1,3,5 years in TCGA-ACC (n=79), GSE10927 (n=24), GSE19750 (n=22), GSE76019 (n=34). ACC, adrenocortical carcinoma; BUB1B, BUB1 mitotic checkpoint serine/threonine kinase B; PINK1, PTEN induced kinase 1; OS, overall survival; PFI, progression-free interval; EFS, event-free survival; TCGA, The Cancer Genome Atlas.

As shown in Result 1, we divided the samples into BUB1B-PINK1 high expression group and low expression group by taking the optimal cutoff value. In the TCGA-ACC cohort,

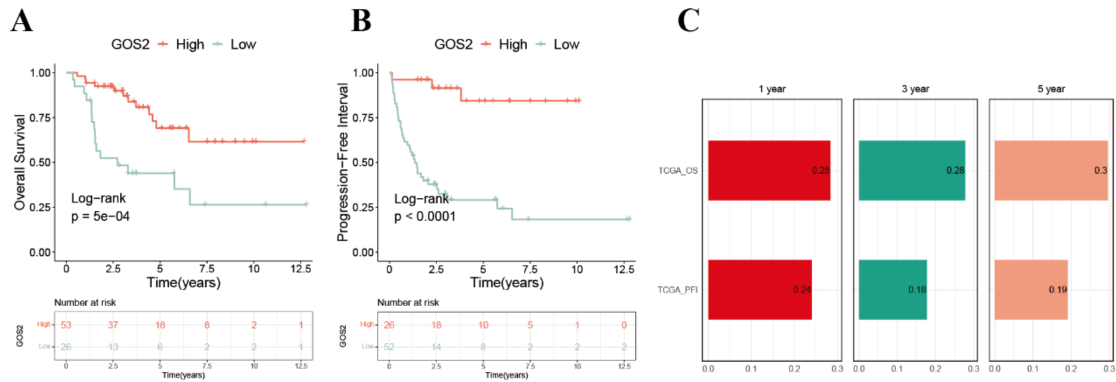
patients who had high BUB1B-PINK1 expression had worse OS and PFI (P<0.001) (Result 1A,1B). Similarly, in the GSE10927, patients with low BUB1B-PINK1 expression group had more satisfactory OS compared to those with high expression (P=0.042) (Result 1C). But, In GSE19750, there was no significant difference in OS between the high and low expression groups of BUB1B-PINK1 (P=0.07) (Result 1D). Additionally, in the pediatric cohort GSE76019, a higher expression of BUB1B-PINK1 was linked to a lower EFS (p=0.001) (Result 1E). ROC curve results showed that in TCGA-ACC, the AUC values for 1, 3 and 5-year OS were 0.79, 0.91, and 0.86, correspondingly, and for 1, 3, and 5-year PFI were 0.75, 0.82 and 0.82. In GSE10927, the AUC values for 1, 3 and 5-year OS were 0.82, 0.64 and 0.06; In GSE76019, the AUC values for 1, 3 and 5-year EFS were 0.65, 0.71 and 0.78 (Result 1F). According to the results, BUB1B-PINK1 provides a strong predictive performance for the prognosis of ACC patients. Using survival analysis and ROC curve comparison, we have identified ZFHX4-AS1 and BUB1B-PINK1 as potential predictors of ACC prognosis. However, our comparative analysis reveals that ZFHX4-AS1 exhibits significantly higher AUC values at 1, 3, and 5 years for ACC patients compared to BUB1B-PINK1. This suggests that ZFHX4-AS1 has superior prognostic predictive capabilities compared to BUB1B-PINK1 in the context of ACC.



Result 2 Survival analysis and performance evaluation of IGF2. (A,C,B,D) Kaplan-Meier survival analysis between the high and low IGF2 groups across TCGA cohorts (OS/PFI/DFI/DSS). IGF2, insulin like growth factor 2; OS, overall survival; PFI, progression-free interval; DFI, disease-free interval; DSS, disease-specific survival; TCGA, The Cancer Genome Atlas.

Using a consistent methodology, we investigated the potential impact of IGF2 on the prognosis of ACC patients. Intriguingly, within the TCGA_ACC cohort, we observed no significant differences in OS, PFI, disease-specific survival (DSS), and disease-free interval

(DFI) between groups with high and low IGF2 expression (P=0.16, P=0.27, P=0.32, P=0.2) (Result 2). Furthermore, there is a lack of chip data related to IGF2 in the GSE10927, GSE19750, and GSE76019 datasets. Upon reviewing existing literature, we noted that IGF2 exhibits the most significant difference in expression levels between normal adrenal gland tissue and adrenal cancer tissue, primarily associated with ACC diagnosis. However, its correlation with the prognosis of ACC patients necessitates further investigation.



Result 3 Survival analysis and performance evaluation of GOS2. (A,B) Kaplan-Meier survival analysis between the high and low GOS2 groups in TCGA-ACC (OS, PFI). (C) Time-dependent ROC analysis for predicting OS/PFI at 1,3,5 years in TCGA-ACC (n=79). ACC, adrenocortical carcinoma; GOS2, G0/G1 switch 2; OS, overall survival; PFI, progression-free interval; TCGA, The Cancer Genome Atlas

As shown in Figure 3, we divided the samples into GOS2 high expression group and low expression group by taking the optimal cutoff value. In the TCGA-ACC cohort, patients who had high GOS2 expression had worse OS and PFI (P<0.001, P<0.001) (Result 3A,3B). Similarly, there is a lack of chip data related to GOS2 in the GSE10927, GSE19750, and GSE76019 datasets. ROC curve results showed that in TCGA-ACC, the AUC values for 1, 3 and 5-year OS were 0.28, 0.28, and 0.30, correspondingly, and for 1, 3, and 5-year PFI were 0.24, 0.18 and 0.19. (Result 3C).

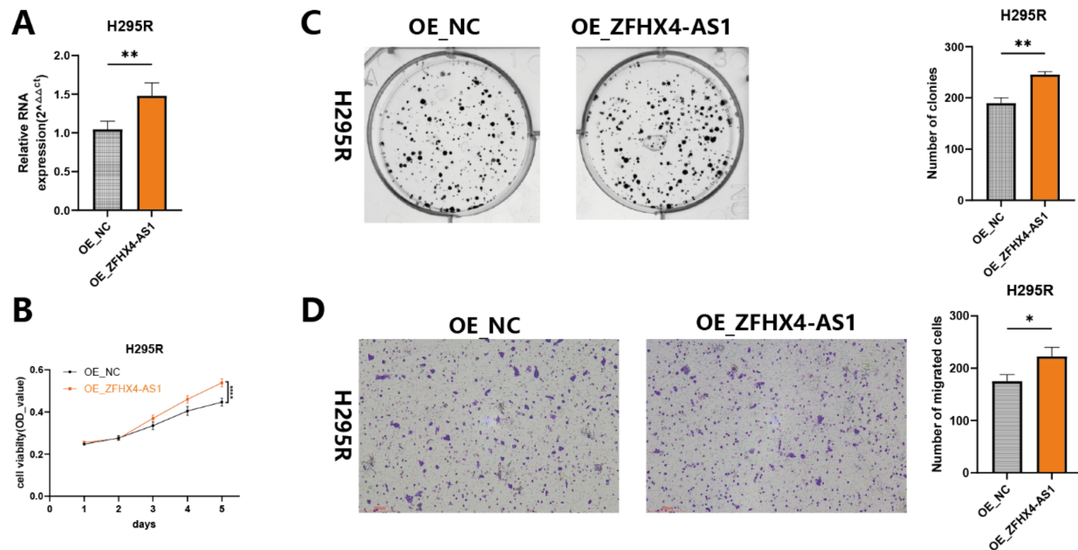
Taken together, these results indicate that ZFHX4-AS1 in this study maintains relatively robust prediction performance even when compared with BUB1B-PINK1, IGF2, and G0S2.

Changes in the text: (see Page 4, line 62-66.)

Comment (3): Is it feasible to conduct an overexpression experiment of ZFHX4-AS1, given the loss-of-function experiments utilizing siRNA by authors. Can the overexpression of

ZFHX4-AS1 induce cellular proliferation in H295R?

Reply (3): Thank you very much for the valuable comments. Our preliminary investigations following ZFHX4-AS1 knockdown revealed its potential to enhance the proliferation and migratory abilities of ACC cell lines. To provide more detailed insights into our experimental findings, we proceeded to overexpress ZFHX4-AS1 and then evaluated its effects on ACC cell lines, particularly H295R. (Experimental methods see Page 7, line 162-170.)



Result 4 Effects of overexpression of lncRNA ZFHX4-AS1 on ACC cell proliferation and migration. (A) The expression of lncRNA ZFHX4-AS1 was significantly increased after overexpression in the H295R cell line. (B,C) CCK-8 assay and plate colony formation assay determine the proliferation ability of H295R cells by overexpressing lncRNA ZFHX4-AS1. (D) Transwell experiment demonstrates the migration ability of H295R cells overexpressed lncRNA ZFHX4-AS1. Error bars represent 95% confidence intervals (CI). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Through in vitro cell experiments involving the knockdown of ZFHX4-AS1 (pCDH-CMV-MCS-EF1-CopGFP-T2A-Puro-ZFHX4-AS1_shRNA), we observed that elevated ZFHX4-AS1 expression can enhance the proliferation and invasion of ACC cells. To further support this finding, we designed specific vectors and verified their transfection efficiency using qRT-PCR. Compared with the control group OE-NC, OE_ZFHX4-AS1 significantly increased the expression of ZFHX4-AS1 in H295R (P=0.01) (Result. 4A). Similarly, CCK-8 experiments showed that overexpression of ZFHX4-AS1 significantly accelerated the proliferation of H295R cells within five days (P<0.001) (Result 4B). Colony formation

experiments suggest that cells overexpressing ZFHX4-AS1 exhibited increased proliferation compared to OE_NC (P=0.01) (Result. 4C). In addition, the Transwell assay confirmed that the migration ability of H295R cells was significantly enhanced after overexpression of ZFHX4-AS1 (P=0.038) (Result 4D). Overall, we demonstrated through knockdown and overexpression experiments that ZFHX4-AS1 plays a promoting role in the proliferation and migration capabilities of ACC. (Experimental results see Page 10, line 237-249.)

Changes in the text: (Page 7, line 162-170. Page 10, line 237-249.)

Comment (4): In Figure 6, a ceRNA network was performed, depicting the mRNAs and miRNAs regulated by ZFHX4-AS1. Following the downregulation of ZFHX4-AS1 in the siRNA experiment, did these mRNAs and miRNAs exhibit the anticipated alterations? It is advisable to validate the results by measuring the expression of selected mRNAs and miRNAs by in vitro experiments.

Reply (4): Thank you for your valuable feedback. We appreciate your concerns, and we are fully aware of them. This article primarily investigates the prognostic predictive capacity and biological functions of the long non-coding RNA (lncRNA) ZFHX4-AS1 in ACC. Our study revealed that ZFHX4-AS1 significantly influences the proliferation, apoptosis, migration, and invasion of ACC cells. Additionally, we have initiated the exploration of the competing endogenous RNA (ceRNA) regulatory network associated with ZFHX4-AS1. However, due to the need to optimize the conditions for comprehensively elucidating the ceRNA network, we have chosen to publish our findings at this stage. We acknowledge that the lack of validation for the ceRNA network represents a limitation of our study. Currently, we are actively engaged in further research in this field and plan to explore it in upcoming experiments. Once again, we sincerely appreciate your valuable feedback.

Comment (5): Incorporating known prognostic factors such as Ki67 and hormone production capacity into the multivariate analysis alongside Age, Gender, and Stage, how would the results be influenced in Table 1?

Reply (5): Thank you for your valuable feedback. Ki67 and hormone production capacity serve as prognostic factors in ACC. In our analysis, we included these factors along with ZFHX4-

AS1 and other clinicopathological variables for both single and multi-factor Cox regression analysis.

Result 5. Univariate and multivariate Cox regression analysis were performed in 4 cohorts.

Characteristics	HR (95% CI)	P value
TCGA_ACC (OS)		
Univariate analysis		
ZFHX4-AS1	1.200 (1.000-1.400)	0.045
MKI67	2.300 (1.700-3.000)	<0.001
CYP11A1	0.850 (0.720-1.000)	0.062
CYP17A1	1.000 (0.890-1.100)	0.880
Age	1.000 (0.990-1.000)	0.260
pN	0.580 (0.170-1.900)	0.370
pT	1.200 (0.820-1.800)	0.330
gender	0.200 (0.089-0.470)	<0.001
Stage	1.100 (0.730-1.700)	0.640
Multivariate analysis		
ZFHX4-AS1	1.100 (0.920-1.300)	0.034
MKI67	2.100 (1.500-2.900)	<0.001
gender	0.740 (0.260-2.100)	0.560
TCGA_ACC (PFI)		
Univariate analysis		
ZFHX4-AS1	1.300 (1.100-1.400)	<0.001
MKI67	1.700 (1.400-2.100)	<0.001
CYP11A1	0.990 (0.840-1.200)	0.930
CYP17A1	1.100 (0.960-1.200)	0.210
Age	1.000 (0.980-1.000)	0.770
pN	0.940 (0.390-2.300)	0.890
pT	1.200 (0.890-1.700)	0.210
gender	0.380 (0.200-0.720)	0.003

Stage	1.200 (0.890-1.700)	0.210
Multivariate analysis		
ZFHX4-AS1	1.300 (1.100-1.400)	<0.001
MKI67	1.800 (1.400-2.300)	<0.001
gender	0.930 (0.460-1.900)	0.850
GSE10927 (OS)		
Univariate analysis		
ZFHX4-AS1	11.000 (2.500-51.000)	0.002
MKI67	2.500 (0.600-10.000)	0.210
CYP11A1	0.270 (0.077-0.950)	0.041
CYP17A1	0.700 (0.360-1.400)	0.310
Age	1.000 (0.960-1.100)	0.640
gender	2.800 (1.000-7.600)	0.044
Multivariate analysis		
ZFHX4-AS1	14.000 (2.700-71.000)	0.002
CYP11A1	0.270 (0.054-1.300)	0.110
gender	2.000 (0.640-6.200)	0.230
GSE19750 (OS)		
Univariate analysis		
ZFHX4-AS1	1.500 (1.000-2.300)	0.039
MKI67	1.600 (1.100-2.200)	0.010
CYP11A1	0.870 (0.700-1.100)	0.190
CYP17A1	0.970 (0.850-1.100)	0.640
Age	1.000 (0.990-1.100)	0.097
gender	0.790 (0.290-2.200)	0.650
stage	1.500 (0.910-2.500)	0.110
Multivariate analysis		
ZFHX4-AS1	1.500 (1.000-2.300)	0.047
MKI67	1.600 (1.100-2.200)	0.012

GSE76019 (EFS)		
Univariate analysis		
ZFHX4-AS1	1.800 (1.200-2.800)	0.006
MKI67	1.600 (1.000-2.400)	0.035
CYP11A1	0.370 (0.210-0.670)	<0.001
CYP17A1	0.840 (0.580-1.200)	0.360
Age	1.30 (1.10-1.400)	<0.001
gender	0.180 (0.053-0.590)	0.005
Stage	3.100 (1.500-6.400)	0.003
Multivariate analysis		
ZFHX4-AS1	1.300 (0.650-2.500)	0.470
MKI67	1.500 (0.880-2.400)	0.140
CYP11A1	0.560 (0.250-1.200)	0.160
Age	1.200 (1.000-1.300)	0.054
Stage	1.800 (0.600-5.400)	0.300

ACC, adrenocortical carcinoma; OS, overall survival; PFI, progression-free interval; EFS, event-free survival; TCGA, The Cancer Genome Atlas. ZFHX4-AS1, ZFHX4 Antisense RNA 1; MKI67, Marker Of Proliferation Ki-67; CYP11A1, Cytochrome P450 Family 11 Subfamily A Member 1; CYP17A1, Cytochrome P450 Family 17 Subfamily A Member 1.

In the Cox regression univariate analysis conducted within the TCGA-ACC cohort, it was found that lncRNA ZFHX4-AS1 and MKI67 were associated with both OS and PFI among ACC patients. Within the GSE10927 cohort, the lncRNA ZFHX4-AS1, CYP11A1, and gender showed significant associations with OS in ACC patients. Subsequent multivariate Cox regression analysis highlighted the significance of ZFHX4-AS1 in relation to patients' OS. Similarly, in the GSE19750 cohort, both univariate and multivariate Cox regression results indicated associations between lncRNA ZFHX4-AS1 and MKI67 with OS in ACC patients. Within the GSE76019 cohort, lncRNA ZFHX4-AS1, MKI67, CYP11A1, age, stage, and gender were identified as relevant factors associated with EFS in ACC patients.In summary, ZFHX4-AS1 and MKI67 emerge as independent prognostic risk factors for ACC patients. Notably, ZFHX4-AS1 demonstrates robust prognostic predictive capabilities across all four cohorts,

establishing it as a biomarker with potent predictive efficacy for ACC prognosis.

Comment (6): Insufficient details are provided regarding the methodology of the transfection experiments. Clarification is required on the transfection method employed, whether it be lipofection or electroporation. Additionally, it is essential to specify the duration between transfection and assay execution.

Reply (6): Thank you for your valuable feedback. The method employed in our study is lipofection, with a transfection duration of 72 hours. We have thoroughly reviewed and updated the experimental methods section in our manuscript to include detailed descriptions of the procedure.

Changes in the text: (see Page 7, line 153-161.)

Reviewer B

Comment (1): In the abstract the authors state that ZFXH4-AS1 "…plays a significant role in cancer growth and metastasis". However, in the Introduction, the authors describe in detail other ncRNAs, their roles and mechanism of action, however, the statements they make about the main subject of the paper, ZFXH4-AS1, are rather vague.

Reply (1): Thank you for your valuable feedback. We are aware of this issue, and there are currently relatively few studies on ZFHX4-AS1. Therefore, we have made an effort to describe it as comprehensively as possible.

ZFHX4 is one of the five members of the zinc finger homeobox family, with a molecular weight of 397 kDa. This protein contains four homologous domains and 22 zinc finger structures, located at 8q13.3-q21.1. ZFHX4-AS1 is a newly discovered lncRNA that targets the antisense sequence of the ZFHX4 gene, located on chromosome 8q21.13. There are few reports on the functional study of ZFHX4-AS1 in tumors. ZFHX4-AS1 was initially identified as significantly overexpressed in breast cancer, promoting cell apoptosis by inhibiting the Hippo signaling pathway. Additionally, it is currently recognized as a prognostic marker for ovarian cancer, breast cancer, and bladder cancer, predicting the prognosis of tumor patients. Studies have shown its association with thyroid-associated ophthalmopathy, gastric cancer, and susceptibility to glaucoma. Furthermore, it can function as a competing endogenous RNA

(ceRNA) to influence the severity of schizophrenia.

Changes in the text: (see Page 11, line 294-303.)

Comment (2): While the fact that expression of ZFXH4-AS1 correlates with poor survival is interesting, the way the in silico experiment is conducted, it is unclear, what does this mean? Cancer cells are known to be highly proliferative and to contain a proliferative gene signature (E.g. E2F1, MYC, EZH2 etc.). If ZFXH4-AS1 is a part of this cluster, tracking its expression is no worse and no better than tracking any other gene in this cluster.

Reply (2): Thank you for your insightful feedback. We appreciate your interest in this aspect of our research. Indeed, the correlation between ZFXH4-AS1 expression and poor survival is intriguing. At present, bioinformatics holds a pivotal position in the field of medicine. Integrating diverse disciplines such as biology, computer science, and statistics, bioinformatics employs computational and analytical approaches to manage and interpret biological data. This interdisciplinary approach has led to significant advancements in various domains, including genomics, proteomics, and systems biology. Our investigation aligns within this realm.In essence, the integration of bioinformatics in medicine provides us with new perspectives and methodologies, enabling the exploration of disease mechanisms, the advancement of diagnostic techniques, and the realization of personalized medicine.

As you rightly pointed out, cancer cells often exhibit proliferative gene signatures, including well-known genes such as E2F1, MYC, and EZH2. Our study aimed to elucidate the specific role of ZFXH4-AS1 in this context. While it is likely that ZFXH4-AS1 is part of this group of proliferation-related genes, our analysis offers valuable insights into its unique contribution to cancer progression and patient outcomes. Through tracking the expression of ZFXH4-AS1 along with other genes in this cluster, we aimed to determine if ZFXH4-AS1 provides unique prognostic value beyond established proliferation markers. Our study investigated the potential of ZFXH4-AS1 as a novel biomarker for predicting cancer prognosis. Moving forward, we plan to further elucidate the regulatory mechanism of ZFXH4-AS1 in ACC through the ceRNA network and comprehensively explore its biological functions. Once again, we sincerely appreciate your comments and look forward to addressing them in our future work.

Comment (3): The in vitro experiments appear not well controlled. There is no control siRNA containing a mutation that should serve as a negative control; in my opinion, that is a major flaw.

Reply (3): Thank you for your valuable feedback. We fully understand your concerns. In accordance with standard research protocols, we designed three siRNAs targeting ZFHX4-AS1 and transfected them stably using the RNAi-Mate reagent. The complexes formed with siRNAs were co-cultured for 72 hours. We validated the transfection efficiency of siRNAs using quantitative reverse transcription polymerase chain reaction (qRT-PCR). Once again, we appreciate your insightful feedback.

Changes in the text: (see Page 7, line 153-161.)

Comment (4): It is also not clear, if the authors are arguing that ZFXH4-AS1 is important in every single cell line? If not, the authors should show that their primers have no effect in a cell line that does not depend on ZFXH4-AS1.

Reply (4): We greatly appreciate your valuable feedback. We fully understand your concerns. In accordance with standard research protocols, we designed three siRNAs targeting ZFHX4-AS1. (si-ZFHX4-AS1#1:UAUGAUAGACAUUUCUACC; si-ZFHX4-AS1#2:UAGUAAUGUUGUUACAUCC; si-ZFHX4-AS1#3:UGUAACAUUAUGAAUCUGG). We adopted a widely utilized protocol and used two types of ACC cells (H295R and SW-13) for our in vitro experiments. Our findings indicate that ZFHX4-AS1 enhances the proliferation and migration of ACC cells. Furthermore, we substantiated this conclusion by overexpressing ZFHX4-AS1 (Page 7, line 162-170. Page 10, line 237-249.).

Changes in the text: (Page 7, line 162-170. Page 10, line 237-249.)

Reviewer C

Comment (1): The more clinicopathologic details regarding prognosis between high and low ZFHX4-AS1 expression groups, including stage, may consider to provide.

Reply (1): Thank you for your valuable feedback. To enhance the credibility of our Cox regression results, we included established prognostic factors such as Ki67 and hormone

production capacity markers (CYP11A1, CYP17A1), in addition to ZFHX4-AS1 and other clinicopathological variables. These factors were collectively subjected to both univariate and multivariate Cox regression analyses.

In the Cox regression univariate analysis conducted within the TCGA-ACC cohort, it was found that lncRNA ZFHX4-AS1 and MKI67 were associated with both OS and PFI among ACC patients. Within the GSE10927 cohort, the lncRNA ZFHX4-AS1, CYP11A1, and gender showed significant associations with OS in ACC patients. Subsequent multivariate Cox regression analysis highlighted the significance of ZFHX4-AS1 in relation to patients' OS. Similarly, in the GSE19750 cohort, both univariate and multivariate Cox regression results indicated associations between lncRNA ZFHX4-AS1 and MKI67 with OS in ACC patients. Within the GSE76019 cohort, lncRNA ZFHX4-AS1, MKI67, CYP11A1, age, stage, and gender were identified as relevant factors associated with EFS in ACC patients.In summary, ZFHX4-AS1 and MKI67 emerge as independent prognostic risk factors for ACC patients. Notably, ZFHX4-AS1 demonstrates robust prognostic predictive capabilities across all four cohorts, establishing it as a biomarker with potent predictive efficacy for ACC prognosis (Result 5).

Comment (2): Please provide how to define high and low ZFHX4-AS1 expression group.

Reply (2): Thank you for your valuable feedback. In accordance with standard research practices, we used survminer backage to determine the optimal cutoff value. Subsequently, patients with ACC were stratified into two groups based on their ZFHX4-AS1 expression levels: high expression and low expression.

Changes in the text: (Page 6, line 125-127.)

Comment (3): Thorough text checking to correct grammatic errors is needed.

Reply (3): Thank you for your valuable feedback. We recognize the importance of ensuring grammatical accuracy in our manuscript. We will conduct a thorough review of the text to correct any grammatical errors before resubmitting it.