Response to the Reviewer #1

Comment (1): the authors should guide the readers to the meaning of the images appropriately; otherwise, it is likely to cause misunderstandings.

Response: Thanks for the suggestion. We have revised accordingly.

Comment (2): In Fig2A suggested that several cell cycles associated genes were up-regulated in CCA cancer lines, including PCNA, E2F1, CDK2, CDK4, and CDK6. Since the authors gave a general answer on gene expression, is there any evidence of different roles in cancer phenotypes of these genes? Please perform pertinent bioinformatic analyses and provide examples of studies investigating miRNA alteration or DNA methylation.

Response: Thanks for your suggestions. We performed the correlation analysis between the expressions of those genes and their methylation status (β value). As shown in Figure S1, the expression of JAG1, JAG2, and ATR are positive correlated with their methylation statuses, which indicated other regulation should be responsible for the aberrant expression of these genes. In line with the gene regulation controlled by DNA methylation, the expression of other genes shown in Figure S1 were negatively correlated with their corresponding β value, suggesting that the aberrant expression of the other genes are highly possible due to the DNA methylation epigenetic modification.

Comment (3): So far, the tumor infiltrates immune cells and is vital for patient survival. However, it is worth validating their data correlated with immune cells by using the "TIMER" (http://timer.cistrome.org) analysis tool.

Response: We appreciate this comment. We did a similar analysis by following the previous study published by Charoentong et. al [1]. Single-sample GSEA (ssGSEA) was utilized to analyzed the tumor-infiltrating lymphocytes (TILs) based on the 28 immune cell types. As shown in Figure S4, the cell cycle associated pathways are close correlated with the levels of TILs. Those correlations may indicate the cancer immuno-therapy.

[1] Charoentong P, Finotello F, Angelova M, et al. Pan-cancer immunogenomic analyses reveal genotype-immunophenotype relationships and predictors of response to checkpoint blockade[J]. Cell reports, 2017, 18(1): 248-262.

Comment (4): Since Connectivity Map (CMap) can be used to discover the mechanism of action of small molecules, functionally annotate genetic variants of disease genes, and inform clinical trials. It would be fascinating if these data could be correlated with other clinical databases. Therefore, I suggest the authors can validate their data via CMap or proteinatlas, and discuss these methodologies and literature as well as the validated data for cancer recurrence or metastasis in the manuscript.

Response: Thanks for your comments. The mode of Gene Expression (L1000) [2] was performed based on various cancer cell lines (240) except cholangiocarcinoma. Besides,

the human protein atlas (HPA) [3] does not have the pathology of cholangiocarcinoma either. We will focus on those kinds of databases and do analysis in the future.

[2] Subramanian A, Narayan R, Corsello S M, et al. Asiedu JKet al. 2017[J]. A next generation connectivity map: L1000 Platform and the first, 1: 1437-1452.
[3] Chin C H, Chen S H, Wu H H, et al. cytoHubba: identifying hub objects and sub-networks from complex interactome[J]. BMC systems biology, 2014, 8(4): 1-7.

Comment (5): The author should use other statistical analyses such as ANOVA to calculate the P-value for three or more groups of data, and please update the “Statistical Analysis” of the Method during further revision. For example, please add the correct P-value for Figure 2. Same as Figure 3, please also perform statistical analysis for these data.

Response: Thank you for highlighting this issue. We revised the “Statistical methodologies” of the Method of the manuscript. The P-value was also added to the figures.

Comment (6): There are few typo issues for the authors to pay attention to; please also unify the writing of scientific terms. “Italic, capital”? For example, Jag1, Jag2 in Page 6, and Italic form of JAG1/JAG2 in page 7.

Response: Thank you for the precious suggestion. We have unified the style of scientific terms.

Comment (7): The font is too small for some of the current figures, meanwhile, the manuscript also needs English proofreading.

Response: Thanks for the comments. We changed the font size as requested. Besides, we have asked two native English speakers who major in biomedical science to proofread our manuscript.

Response to the Reviewer #2:

Comment (1): Previous studies have reported the role of cell cycle and Notch in CCA, which the author has found these two indicators via the database and also verified them in this paper. The author is requested to supplement the previous studies as well as the innovation of this paper in the discussion.

Response: Thanks for the comment. We cited the previous studies and discussed innovation in the current manuscript.

Comment (2): In the Preface, the author states that "it is in urgent need to develop early detection markers and effective treatment methods for CCA patients". Do the cell cycle and Notch, which are the focus of this paper, change at the early phase of tumor formation? The author is requested to supplement relevant content in the discussion.

Response: We have added the relevant discussion.

Comment (3): The internal reference GAPDH was chosen for the detection of cell line mRNA in Figure 2, while different internal references, i.e. α-Tubulin and GAPDH were chosen for the protein detection of cell line in Figure 3 and mouse tissue in Figure 4. The author is requested to explain the reasons and provide the original pictures of WB.

Response: We apologize for the issue. We used 18S rRNA as the internal control for qPCR experiments. We used equal amount of mRNA and protein for the qPCR and western blot experiments. In this case, we should see equal amount of internal control. However, we did observe some difference. After testing other internal controls, we found the relevant controls can better reflect the equal amount of mRNA and protein loading. In this case, we chose those internal controls for our data interpretation. We also provide the original pictures of western blot in the revised manuscript.

Comment (4): Do the P53null/KRasG12D gene editing mice used in Figure 4 spontaneously form tumors, and what is the age of the chosen diseased mice? The author is requested to supplement this content.

Response: The genomic engineer mouse model (GEM) was established by breeding floxed P53, Albumin Cre, and KrasG12D mice to obtain the harbored P53null/$KRas^{G12D}$ mutations. This model was initially created by O'Dell et. al [4], which cab be utilized to closely recapitulated the multistage histopathologic progression of the intra-hepatic cholangiocarcinoma (iCCA), including the development of stroma-rich tumors, the premalignant biliary lesions, intraductal papillary biliary neoplasms (IPBN), and Von Meyenburg complexes. This GEM combining $Kras^{G12D}$ activation with homozygous deletion of P53 would cause the spontaneously formed tumors. The mice are 52 weeks old. We have added the information to the revised manuscript.

[4] O'Dell M R, Li Huang J, Whitney-Miller C L, et al. KrasG12D and p53 Mutation Cause Primary Intrahepatic CholangiocarcinomaKras and p53 Cooperate to Cause Intrahepatic Cholangiocarcinoma[J]. Cancer research, 2012, 72(6): 1557-1567.

Comment (5): There is no normal cell line control in the experiment of detecting the cytotoxicity of inhibitors of cell cycle and Notch pathways to CAA in Figure 5. Notch is associated with cell proliferation, and cell proliferation can be apparently inhibited by inhibiting cell cycle or Notch, so it is requisite to distinguish the differences between these inhibitors and normal cells.

Response: The reviewer gave an excellent comment that cell cycle and Notch pathways are required for the normal cell proliferation. We did observe that both cell cycle and Notch inhibitors inhibited cell proliferation in normal bile duct cells. Since these inhibitors have been well studied regarding cytotoxicity tolerance in animal models, we did not evaluate their toxic effects on the GEM model. To avoid confusion to the reader, we did not include the data of normal bile duct cells in this study.

Comment (6): The author searched for the key biological indicators associated with CAA by means of bioinformatics, and then focused on cell cycle as well as Notch gene, by contrast, P53null/KRasG12D gene editing mice was used in the mouse model. It was proved that this model is similar to human CAA and is a potential pre-clinical CCA model. It seems that the changes of P53 and KRas precede the changes of cell cycle and Notch, then why not P53 and KRas are chosen as the biological indicators of CAA directly for the purpose of treatment?

Response: Thanks for the comment. The purpose of understanding the molecular mechanisms in tumorigenesis is to identify the potential downstream target. Accumulating evidence has indicated that targeting the upstream may not cure CCA patients, such as IDH1 mutation inhibitor [6]. In this case, we must further clarify the underlying molecular pathogenesis of CCA.

[6] Andrew X. Zhu, MD, PhD1,2; Teresa Macarulla, MD3; Milind M. Javle, MD4; et al. Final Overall Survival Efficacy Results of Ivosidenib for Patients With Advanced Cholangiocarcinoma With IDH1 MutationThe Phase 3 Randomized Clinical ClarIDHy Trial. JAMA Oncol. 2021;7(11):1669-1677. doi:10.1001/jamaoncol.2021.3836

Response to the Reviewer #3:

Comment (1): Given the natural product origin of both small molecule inhibitors (Arcyriaflavin and Flavopiridol) it is recommended to add a short paragraph to introduce GENERAL health-promoting benefits of natural products.

Response: Thanks for the comment. Since the study is focused on the molecular pathogenesis, we do not include the benefits of natural products in the current manuscript.

Comment (2): Proofreading is required.

Response: We have asked two native English speakers who major in biomedical science to proofread our manuscript.

Comment (3): Uncropped gels for all blots should be made available as supplementary data.

Response: The original pictures of WB were supplied.

Comment (4): All western blots must be quantified and properly analyzed.

Response: Thank you for the comment. We have quantified the western blot results. The data is provided in Figure S2 and Figure S3.

Comment (5): Figure 2 lacks proper statistical analysis.

Response: Thank you for highlighting this issue. We have revised the “Statistical methodologies” of the Method of the manuscript. The P-value was also added to the figures.

Comment (6): Figure 4D image and its legend should have labels to clarify relevant lesions.

Response: We have added the arrow to indicate lesions.

Comment (7): A conclusion figure illustrating how cell cycle and notch pathways contribute to CCA should be added.

Response: Thanks for your suggestion. The conclusion is presented in the Figure 8.

Comment (8): References should be enriched with more diversified investigations. Results from the following studies could serve this purpose: https://doi.org/10.1186/s41936-022-00321-7, PMID: 36432184, PMID: 35740022.

Response: Thanks for the suggestion. We included some references accordingly.