Genes & Diseases (2022) 9, 1427–1430

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: http://ees.elsevier.com/gendis/default.asp

RAPID COMMUNICATION

Regulation of pancreatic cancer metastasis through the Gli2-YAP1 axis via regulation of anoikis

Pancreatic cancer, mostly pancreatic ductal adenocarcinoma (PDAC), is often metastatic upon the initial diagnosis, and has a high mortality rate. However, the primary and the matched metastatic cancers often share the same driver gene mutations.[1,2] Gene expression comparison between primary and metastatic PDAC tissues revealed high expression of Gli2 and YAP1 in the metastatic PDAC. Gli2 is an important player in non-canonical hedgehog signaling, and YAP1 is an essential cell polarity gene. In this study, we investigated the effects of Gli2 and YAP1 on pancreatic cancer cell function in 3D culture and in mouse models. We further determined the relationship between Gli2 and YAP1. These studies further our understanding of pancreatic cancer metastasis.

Using Gene Expression Omnibus (GEO) datasets, we identified several genes highly expressed in metastatic PDAC, including *Gli2* and *YAP1* (Table S1). To understand how Gli2 and YAP1 are involved in the metastatic process, we tested whether Gli2 knockdown affects pancreatic cancer cell function. First, we determined whether Gli2 affects anoikis in model systems. The cells were plated on ultra-low attachment plates and incubated for 48 h at 37 °C. As shown in Figure 1A, the control cells formed large tumorspheres, while Gli2 knockout cells formed much smaller spheres (0.497 ± 0.017 for ASPC1/CRISPR/Gli2 and 0.640 ± 0.037 for PANC1/CRISPR/Gli2 in diameters respectively, control groups referred as 1.0, $P < 0.001$). The rates of anoikis in the control and Gli2 knockout cells in suspension were then detected by EthD1 staining followed by flow cytometry.[3] We found that the rates in Gli2 knockout cells were significantly higher than those in control cells (23.87% ± 1.27% *vs.* 11.53% ± 0.65% for ASPC1, $P = 0.003$; 19.57% ± 0.75% *vs.* 6.65% ± 0.39% for PANC1, $P < 0.001$; Fig. 1B). Since cleaved caspase-3 indicates cell death, we examined the protein level of cleaved caspase-3 in anoikis cells. As expected, cleaved caspase-3 was increased in Gli2 knockout cells (ASPC1/CRISPR/Gli2 and PANC1/CRISPR/Gli2 cells, Fig. 1C). These data indicate that Gli2 knockout in pancreatic cancer cells induces anoikis.

Next, we injected 5×10^5 Gli2 knockout cells (GFP-infected ASPC1/CRISPR/V2 or ASPC1/CRISPR/Gli2) into the tail vein of immunodeficient NOD/SCID Gamma mice mice. Anoikis was shown as the percentage of cleaved caspase-3 positive cells in all GFP positive cells. As shown in Figure 1D, the ASPC1/CRISPR/Gli2 expressing lung tissues displayed more cleaved caspase-3 positive cells, as high as 72.4%. In contrast, cells without Gli2 knockout (ASPC1/CRISPR/V2) had less cleaved caspase-3 positive cells (~40%, see Fig. 1E).

As another significant change in metastatic PDAC, we examined YAP1 protein following Gli2 knockout. We found that YAP1 phosphorylation is decreased in ASPC1 and PANC1 cells after Gli2 knockout, and the phospho-YAP1/total YAP1 ratio was elevated to 2.603 ± 0.261 and 1.721 ± 0.151, respectively, as indicated by Western blot analysis (Fig. 1F). Furthermore, immunofluorescence staining showed the increased cellular localization of YAP1 in Gli2 knockout cells compared to the control cells (Fig. 1G). These results suggest that YAP1 activity is decreased in Gli2 knockout pancreatic cancer cells.

To determine whether the phenotypes of Gli2 knockout pancreatic cancer cells were functionally caused by YAP1 inactivation, Gli2 knockout cells (ASPC1/CRISPR/Gli2 and PANC1/CRISPR/Gli2) were ectopically expressed with a constitutively active form of YAP1, $YAP1^{5SA}$.[4] The five Ser residues were replaced with Ala in $YAP1^{5SA}$, which caused YAP1 sustained activation. Successful transfection of $YAP1^{5SA}$ was verified by detecting Flag in transfected cells (Fig. 1H). Indeed, when $YAP1^{5SA}$ was introduced into ASPC1/CRISPR/Gli2 and PANC1/CRISPR/Gli2 cells, the inhibition effect of Gli2 knockout on tumorsphere size was reversed

Peer review under responsibility of Chongqing Medical University.

https://doi.org/10.1016/j.gendis.2022.05.010

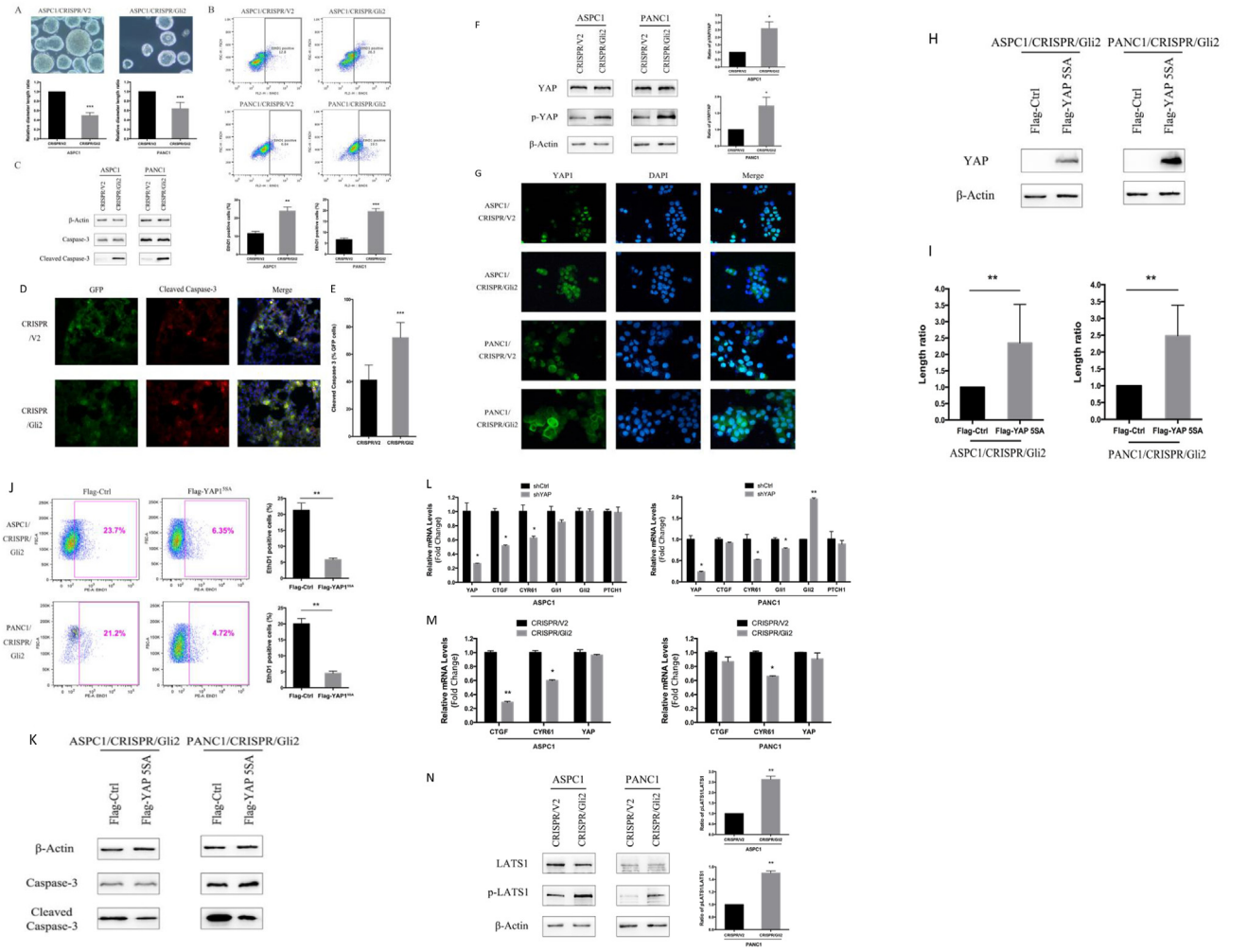

Figure 1 The role of Gli2 and YAP1 in regulation of pancreatic cancer cell anoikis. **(A—C)** Gli2 knockout in pancreatic cancer cells induces anoikis. Representative photographs of tumor spheres in ASPC1 Gli2 knockout cells and their control cells after 48 h of culture in suspension. The bar graph shows the mean diameter length ratio of tumor spheres in Gli2 knockout cells compared with corresponding control cells (ASPC1 and PANC1) (A). Representative histograms depicting apoptosis and the apoptosis rates of ASPC1 and PANC1 (Gli2 knockout and control) cells after 48 h of culture in suspension (B). Protein expression of caspase-3 and cleaved caspase-3 in Gli2 knockout cells and control cells by Western blot analysis. Data are represented as mean ± SD from three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ (C). **(D, E)** Knockout of Gli2 induces anoikis *in vivo*. Representative images of cleaved caspase-3 and GFP positive cells in mouse lungs 24 h after injection with ASPC1/CRISPR/V2 or ASPC1/CRISPR/Gli2 cells. Blue: 4′-6-diamidino-2-phenylindole (DAPI); Green: GFP; Red: cleaved caspase-3 (D). The bar graph shows the mean percentage of cleaved caspase-3 positive cells in GFP positive cells in lungs from ASPC1/CRISPR/V2 or ASPC1/CRISPR/Gli2 injected mice. Data are represented as mean ± SD. $^{***}P < 0.001$ (E). **(F, G)** Gli2 knockout induces anoikis and YAP1 inactivation in pancreatic cancer cells. Western blot analysis shows total YAP1 and phosphorylated YAP1 in ASPC1 and PANC1 (Gli2 knockout and the control) cells. The bar graph represents the ratio of phosphor-YAP1/YAP1. Data are means ± SD of triplicates. $^{*}P < 0.05$ (F). Immunofluorescence staining for YAP1 in CRISPR/V2 and CRISPR/Gli2 of ASPC1 and PANC1 cells (G). **(H—K)** Ectopic expression YAP1 rescues the Gli2 knockout effect in pancreatic cancer cells. Western blot analysis of YAP1 in ASPC1/CRISPR/Gli2 and PANC1/CRISPR/Gli2 cells with expression Flag-Ctrl and Flag-YAP1^{5SA} cells, with their parental cells used as negative controls (H). Bar graphs of tumor spheres in ASPC1/CRISPR/Gli2 and PANC1/CRISPR/Gli2 with the expression of YAP1^{5SA} compared with the control cells after 48 h of culture in suspension (I). Representative histograms depicting apoptosis in ASPC1/CRISPR/Gli2 and PANC1/CRISPR/Gli2 with the expression of YAP1^{5SA} after 48 h of anoikis induction. The apoptosis rates of the two groups of cells were shown (J). Protein expression of caspase-3 and cleaved caspase-3 in YAP1^{5SA} overexpression cells compared with the control cells by Western blot analysis. Data are shown as mean ± SD from three independent experiments. $^{**}P < 0.01$ (K). **(L—N)** Gli2 knockout in pancreatic cancer cells downregulates YAP target genes and LATS1 phosphorylation. Real-time PCR analysis of *YAP1*, *CTGF*, *CYR61*, *Gli1*, *Gli2*, and *PTCH1* transcript levels in ASPC1/shYAP1, PANC1/shYAP1, and their relative control cells (L). Real-time PCR analysis of *CTGF*, *CYR61*, and *YAP1* transcripts in ASPC1 and PANC1 Gli2 knockout cells and their matched control cells (M). Western blot analysis of total LATS1 and phosphorylated-LATS1 in ASPC1 and PANC1 Gli2 knockout cells and their control cells. The bar graph shows the ratio of phosphor-LATS1/LATS1. Data are represented as mean ± SD of triplicates. $^{*}P < 0.05$, $^{**}P < 0.01$ (N).

(2.347 ± 0.372 and 2.482 ± 0.323 in diameters, respectively, all control groups also referred to as 1.0, $P < 0.01$, Fig. 1I). Moreover, progression toward anoikis was also reduced when YAP1^{5SA} was expressed in Gli2 knockout cells (ASPC1/CRISPR/Gli2 and PANC1/CRISPR/Gli2) (5.85% ± 0.27% *vs.* 21.30% ± 1.31% for ASPC1, $P = 0.0055$; 4.53% ± 0.38% *vs.* 20.00% ± 0.96% for PANC1, $P = 0.0013$, Fig. 1J). Confirming the effect, the protein level of cleaved caspase-3 was dramatically reduced after YAP1^{5SA} overexpression (Fig. 1K). These data support that anoikis induced by Gli2 knockout is YAP1 expression-dependent.

Next, we determined the mechanism by which Gli2 regulates YAP1. As shown in Figure 1L, YAP1 and YAP pathway target genes *CTGF* and *CYR61* were downregulated in YAP1 knockdown ASPC1 and PANC1 cells. In contrast, the Hedgehog pathway target genes *Gli1*, *Gli2*, and *PTCH1* were not affected by YAP1 knockout. However, Gli2 knockout reduced YAP1 target genes *CTGF* and *CYR61* (Fig. 1M). These results indicate that Gli2 regulates YAP1, not the reverse order, in pancreatic cancer cells. We further examined the upstream target of the YAP1 pathway by which Gli2 knockout affects. We found that LATS1 phosphorylation was significantly increased after Gli2 knockout in pancreatic cancer cells (Fig. 1N), suggesting that Gli2 knockout induces anoikis by regulation of LATS1 phosphorylation.

The exact mechanisms by which Gli2 regulates LATS1 phosphorylation is being actively investigated, and we have evidence to suggest that phosphorylation of ERK1/2 and AKT phosphorylation is altered by Gli2, and inhibition of MEK1 and AKT1 increases YAP1 phosphorylation. We previously observed that the Erk1/2 pathway was activated by Hedgehog signaling.[5] Western blot analysis showed that Erk1/2 and Akt phosphorylation was markedly decreased in ASPC1 and PANC1 cells by Gli2 knockout (Fig. S1A). Further, MEK1 (upstream of Erk1/2) inhibitor AZD6244 and PI3K (upstream of Akt) inhibitor BKM120, that block these two pathways, increased phosphorylation of YAP and LATS1 (Fig. S1B). Reduced phosphorylation of Erk1/2 and Akt1 by the corresponding inhibitors was associated with an elevated level of cleaved caspase-3 (Fig. S1C), and more anoikis (Fig. S1D). These data suggest that Gli2 knockout induces anoikis by regulation of YAP1 via the ERK1/2 and AKT signaling in metastatic pancreatic cancer cells (as shown in our working model in Fig. S1E).

In summary, we identified *Gli2* and *YAP1* as major upregulated genes during pancreatic cancer metastasis. Knocking down Gli2 induces anoikis in pancreatic cancer cells in 3D culture and in mouse models. Gli2 also regulates cytoplasmic localization of YAP1. Expression of a constitutively active YAP1, YAP1^{5SA}, reversed Gli2-induced effects on anoikis but did not affect Gli2 expression. These results indicate that the Gli2/YAP1 signaling axis is essential for resistance to anoikis during pancreatic cancer metastasis. We predict that down-regulation of the Gli2/YAP1 signaling axis will reduce pancreatic cancer metastasis.

Author contributions

J. Xie and B. Liu initiated and supervised the research. B. Yu and D. Gu performed the majority of the experiments and data analyses. X. Zhang assisted with the experiment work and provided crucial suggestions. B. Yu and J. Xie prepared the manuscript. All authors have reviewed and approved the final version of this manuscript.

Conflict of interests

Authors declare no conflict of interests.

Funding

This work was supported by The Wells Center for Pediatric Research, Riley Children Foundation, Jeff Gordon Children's Foundation, and IU Simon Cancer Center. This work was funded by grants from the National Natural Science Foundation of China (No. 81902393 and 82072605), Natural Science Foundation of Shanghai (No. 19ZR1431700), and the Interdisciplinary Program of Shanghai Jiao Tong University (No. YG2019QNB23).

Data availability

The data sets generated and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2022.05.010.

References

1. Gao J, Aksoy BA, Dogrusoz U, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci Signal*. 2013;6(269):pl1.
2. Cerami E, Gao J, Dogrusoz U, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov*. 2012;2(5):401—404.
3. Hu B, Zhang T, An HM, Zheng JL, Yan X, Huang XW. Herbal formula YGJDSJ inhibits anchorage-independent growth and induces anoikis in hepatocellular carcinoma Bel-7402 cells. *BMC Compl Alternative Med*. 2018;18(1):17.
4. Zhao B, Ye X, Yu J, et al. *TEAD* mediates *YAP*-dependent gene induction and growth control. *Genes Dev*. 2008;22(14):1962—1971.
5. Gu D, Lin H, Zhang X, et al. Simultaneous inhibition of MEK and hh signaling reduces pancreatic cancer metastasis. *Cancers*. 2018;10(11):403.

Beiqin Yu [a,b,1], Dongsheng Gu [b,1], Xiaoli Zhang [b], Bingya Liu [a,**], Jingwu Xie [b,*]

[a] *Department of General Surgery, Shanghai Key Laboratory of Gastric Neoplasms, Shanghai Institute of Digestive Surgery, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, PR China*
[b] *Departments of Pediatrics, Biochemistry and Molecular Biology, Pharmacology and Toxicology, The Wells Center for Pediatrics Research, Indiana University School of Medicine, Indianapolis, IN 46202, USA*

*Corresponding author. Wells Center for Pediatric Research and IU Simon Cancer Center, 1044 West Walnut Street, Room R4-327, Indianapolis, IN 46202, USA. Fax: +1 317 274 8679.

**Corresponding author. No.197 Ruijin Er Road, Shanghai 200025, PR China. Fax: +86 21 64373909.
E-mail addresses: liubingya@sjtu.edu.cn (B. Liu), jinxie@iu.edu (J. Xie)

9 May 2022
Available online 24 May 2022