nature portfolio

Peer Review File

Cholecystectomy-related gut microbiota dysbiosis exacerbates colorectal tumorigenesis

Corresponding Author: Professor Shiming Yang

Parts of this Peer Review File have been redacted as indicated to remove third-party material.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this manuscript, the authors report changes in gut microbiota and in bile acid metabolism following cholecystectomy, both in patients and mice. They show that cholecystectomy increases colorectal carcinogenesis (CRC) in the azoxymethane (AOM) and dextran sodium sulfate (DSS) mouse model, and that this effect is reproduced by some of the changes in gut microbiota, i.e. a decrease in Bifidobacterium breve or an increase in Ruminococcus gnavus as observed in mice, and by an increase in glycol-ursodeoxycholic acid (GUDCA), as observed in humans. They attribute this effect to an inhibition of FXR and its interaction with -catenin, which is reversed by obeticholic acid. Despite its interest, the work raises important concerns.

1. The causal relationship between cholecystectomy and colorectal cancer (CRC) or other complications in humans is still debated, whereas it is presented as well established (lines 346-351).

2. The information regarding the patients provided in Table S1, is very limited so that there could be many confounding factors notably metabolic factors, which could explain the differences in gut microbiota and bile acid profile between the two groups, besides cholecystectomy. The time elapsed since cholecystectomy apparently ranges between 1 and 20 years (lines 462-463), which makes the study group very heterogeneous, considering the adaptation of bile acids metabolism which may occur in patients post-cholecystectomy. Two patients in the group of cholecystectomy developed CRC, whereas it is not specified if CRC occurred before or after cholecystectomy, and how long after if this the case. In addition, the statistic method used to analyze patients'date should appear in the legend of Table S1.

3. The mechanism whereby cholecystectomy promotes CRC in mice was investigated by performing GUDCA gavage, whereas it is TUDCA which increases after cholecystectomy in mice. Likewise, whereas B. breve is decreased following cholecystectomy in mice, it seems to be increased in humans (Fig. 1C). Generally, figure legends are not self-explanatory, which makes the interpretation of data difficult.

4. The English syntax needs to be thoroughly revised in particular to express quantitative data properly, e.g. "more severe tumor distribution" (lines 355-356) does not mean anything.
The meaning of several other sentences is unclear, for example, lines 133 and 147: "tumor distribution…larger and wider"; line 147 "colorectal tumor distribution…greater ; line 200 more obvious CRC phenotype; line 393 "a major role in this process": which process does this refer to?; lines 396-397, "the transformation from CDCA to UDCA …has proven feasible"; lines 432-433 "Our results showed that the combination….to the control group". Line 432, reference 33 is not appropriate.

5. The number of replicates is not indicated for all quantitative data in figure legends. Details are also lacking regarding the histopathological analysis of the colon in mice. The score was the average of scores determined by two experts. Information is lacking regarding the intra- and inter-observer variability in this scoring analysis? Information is also lacking regarding the distribution of lesions along the colon, i.e., proximal vs. distal. How were the different grades of histological severity found in each mouse take into account. In addition, it is not specified which part of the colon or if the entire colon were used for RT-PCR analyses.

6. Some statements like “after cholecysctectomy, the enterohepatic circulation of bile acid was accelerated…” (line 115) was not shown in the present study, and if this is the case, the proportion of conjugated bile acids should be decreased rather than increased as found here (line 118).

7. All abbreviations should be defined.

Reviewer #2

(Remarks to the Author)
This study by Tang B. and colleagues is of great interest for understanding the contribution of cholecystectomy to CRC initiation and progression. It also reveals potential drug targets in this specific scenario of CRC initiation. Most of the experiments are well designed and executed.
However, many concerns should be addressed as follows:

MAJOR COMMENTS:

General advice:
The number of mice (n) used in each experiment should be indicated in all figures.

1. Cholecystectomy and intestinal proliferation:
Humans develop tumors in colon or rectum but rarely in small intestine.
It is necessary to quantify with greater detail the proliferation of normal mucosa in control and GBx mouse intestines. Several questions should be answered:
1.1. GBx does affect only the proliferation of small intestinal cells?
1.2. Is it also affecting the proliferation of colon or rectum mucosa?
1.3. Which are the cells with increased proliferation? Enterocytes, stem cells, other lineages?
This characterization will help to understand whether an abnormal increased proliferation rate could be required for tumor initiation from a particular cell of origin.
2. Intestinal permeability:
GBx mice present an increased intestinal permeability. Several questions arise:
2.1. Is it due to a defective cell differentiation in mucosa?
2.2. Is it due to abnormal mucosa absorption physiology?
2.3. Is it due to a defective barrier/cell-to-cell contact monolayer epithelia?
3. Effect of bile acid on bacteria:
B. breve and R. gnavus are both increased in patients with GBx? A quantification of B. breve should be shown. How is possible that B.breve is sensitive and R. gnavus is resistant to Bile acids?
4. Fecal Microbiota Transplantation:
Authors should dissect the contribution of microbiota versus non-microbiota components from donor fecal material when transplanted into mice. For instance, GBx fecal material from patients or mice present a different bile acid composition. Does it affect tumorigenesis in transplanted mice?
5. Wnt pathway:
The activation of Wnt/beta-catenin pathway by GBx or GUDCA is not correctly measured. Wnt activation should be related to NUCLEAR beta-catenin and not total beta-catenin. This applies for western blot analyses or histoligies all along the study (Figure 5, 6 & corresponding supplementary). Most of beta-catenin is accumulated in cell-to-cell contacts in normal and tumor cells. The active pool is that accumulated in the nucleaus as transcriptional coactivator.
5.1. A reporter assay of Wnt/beta-catenin and PXR/beta-catenin transcriptional activity should be done. This means a reporter (luciferase) gene under the control of TCF or PXR response elements transfected in CRC cells treated with GUDCA.
5.2. Better images with magnification to observe nuclear beta-catenin should be shown.
5.3. An intervention proof-of-concept experiment with a Wnt inhibitor such a TNK inhibitor should be done in vivo to confirm is relevant in GBx induced tumorigenesis.
6. OCA therapy:
Obeticholic acid (OCA) is a FXR agonist ligand. How it also induces the expression of FXR gene? Please explain.
Again Wnt pathway activation should be precisely measured by nuclear and no total beta-catenin.
7. FXR/beta-catenin axis:
A mutant version of FXR unable to bind beta-catenin should be used to prove that OCA is unable to induce Wnt target genes. On the contrary, part of the phenotypes indicated could be due to canonical TCF/beta-catenin pathway upon GBx/GUDCA signaling.

MINOR COMMENTS & IMPROVEMENTS:
1. A detailed statistical analysis of CRC risk factors should be compared versus GBx contribution. If this study has previously done it should be referenced and well discussed. This should include the mutational status of the Wnt pathway on its main components: APC, CTNNB1, AXIN1/2, FBXW7, RNF43 and ZNRF3.
2. Testing the capacity of a R. gnavus strain with loss-of-function mutation or deletion of 7beta-HDSH on tumorigenesis will be relevant to prove its role in producing oncogenic metabolite GUDCA.
3. Does GBx reduce the methylation of FXR gene and its consequent increased expression?

4. How GBx promotes the Wnt pathway? Any molecular result has been provided to explain such connection. This would improve the study significantly.

In conclusion, this is a study of interest for the field of CRC. The experiments, figures and text are well designed and clear. However, there are many relevant aspects that should be significantly improved before its publication in Nature Communications.

Reviewer #3

(Remarks to the Author)
In this manuscript, the authors describe changes in microbiota and fecal bile acid composition in post-cholecystectomy (GBx) patients. The authors link these changes to increased tumor formation in two different colorectal cancer (CRC) mouse models and altered FXR/beta-catenin signaling as a major mechanism for GBx-enhanced CRC. There are multiple publications about GBx-induced changes in microbiota and CRC risk (eg. PMID: 35614513 and https://doi.org/10.3389/fmed.2022.1000563). However, the mechanistic aspects of these changes, in particular, the physical interaction of beta-catenin and FXR (PMID 28714273) are not further explored in the manuscript, which we feel are essential to provide conceptual advance and novelty.

A few points to consider:

1) Patients undergoing cholecystectomy are usually suffering from bile duct obstruction (cholestasis) or other medical conditions. Is it possible that prior conditions also affect the incidence of CRC? This should at least be discussed.

2) The authors performed Xeno-FMT to rescue CRC formation (human derived microbiome to mouse). There are major differences in bile acid metabolism and microbiota composition between human and mouse that should be considered.

3) Are reduced FXR expression/increased beta catenin expression accompanied by reduced FXR-beta catenin interaction only specific to tumor tissue or a common feature of GBx colon (non-tumor tissue)?

4) Is GUDCA a major bile acid affecting CRC formation post-GBx at physiological concentrations?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have provided answers, which raise the following comments:

1. The causal relationship between cholecystectomy and colorectal cancer (CRC) or other complications in humans is still debated, whereas it is presented as well established.

In their answer, the authors provide two references, a review on post-cholecystectomy syndrome and meta-analysis on colorectal cancer (CRC), none of which have been included in the introduction of the revised manuscript.

2. The information regarding the patients provided in Table S1, is very limited so that there could be many confounding factors notably metabolic factors, which could explain the differences in gut microbiota and bile acid profile between the two groups, besides cholecystectomy. The time elapsed since cholecystectomy apparently ranges between 1 and 20 years, which makes the study group very heterogeneous, considering the adaptation of bile acids metabolism which may occur in patients post-cholecystectomy. Two patients in the group of cholecystectomy developed CRC, whereas it is not specified if CRC occurred before or after cholecystectomy, and how long after if this is the case. In addition, the statistic method used to analyze patients'date should appear in the legend of Table S1.

No answer was provided regarding the occurrence of CRC before or after cholecystectomy. Moreover, Table S1 shows that the only significant difference between the healthy and cholecystectomy groups according to chi-square test, was more frequent diarrhea in the cholecystectomy group whereas CRC occurrence was not significantly different between the two groups. Importantly, the post-operative time is of 9.08 ± 0.83 without unit, years presumably. It is very surprising that serum bile acids are increased 9 years after cholecystectomy (Fig. 1f) unless the patients have additional liver disease. Therefore, it would be essential to mention the indication of cholecystectomy and the liver status.

3. The mechanism whereby cholecystectomy promotes CRC in mice was investigated by performing GUDCA gavage, whereas it is TUDCA which increases after cholecystectomy in mice. Likewise, whereas B. breve is decreased following cholecystectomy in mice, it seems to be increased in humans (Fig. 1C). Generally, figure legends are not self-explanatory, which makes the interpretation of data difficult.

The authors performed additional experiments showing that TUDCA has the same effect as GUDCA. Yet, if the objective is to show that the effect of GBx is mediated by TUDCA, TUDCA should reproduce the effect of GBx instead of being

combined with GBx.
It is unclear why the increase in R.gnavus in cholecystectomized patients is not shown in Fig. 1, but shown later in Fig. S4g. The presentation of gut microbiota data remains confusing in the text between GBx alone, GBx + AOM/DSS, APC-GBx, and should not be shown as supplementary, but as a full figure of both mice and patients data as in the response to reviewer (Fig. S4F-L). And still, B.breve is increased in humans (fig. 1C)
Figure legends are still not self-explanatory. For example, what lesions does Fig. 3C precisely show.

4. The English syntax needs to be thoroughly revised in particular to express quantitative data properly, e.g. “more severe tumor distribution” (lines 355-356) does not mean anything. The meaning of several other sentences is unclear, for example, lines 133 and 147: “tumor distribution…larger and wider”; line 147 “colorectal tumor distribution…greater ; line 200 more obvious CRC phenotype; line 393 “a major role in this process”: which process does this refer to?; lines 396-397, “the transformation from CDCA to UDCA …has proven feasible”; lines 432-433 “Our results showed that the combination….to the control group”. Line 432, reference 33 is not appropriate.

Language editing by Springer Nature improves reading of the manuscript.

5. The number of replicates is not indicated for all quantitative data in figure legends. Details are also lacking regarding the histopathological analysis of the colon in mice. The score was the average of scores determined by two experts. Information is lacking regarding the intra- and inter-observer variability in this scoring analysis? Information is also lacking regarding the distribution of lesions along the colon, i.e., proximal vs. distal. How were the different grades of histological severity found in each mouse taken into account. In addition, it is not specified which part of the colon or if the entire colon were used for RT-PCR analyses.

All the details provided in the response should be included in the manuscript. The number of replicates should exceed 3. If one goes in one direction, and the other two in the opposite direction, representative of three is almost random. The legend of representative images is misleading, e.g. Fig. 2c, 2f: it would be more appropriate to show carcinoma, HDG and LDG, which are all found in both Sham and GBx, the difference is only quantitative.

6. Some statements like “after cholecysctectomy, the enterohepatic circulation of bile acid was accelerated…” (line 115) was not shown in the present study, and if this is the case, the proportion of conjugated bile acids should be decreased rather than increased as found here (line 118).

This statement has been corrected.

7. All abbreviations should be defined.

There are still undefined abbreviations, e.g., BSH.

In conclusion, the authors addressed several comments, but a focus on the most consistent data would be more convincing to propose a mechanism, whereby cholecystectomy may promote colorectal cancer .

Reviewer #3

(Remarks to the Author)
The authors have conducted a significant number of experiments that have substantially improved the overall quality of their manuscript. However, a few critical points remain to be addressed before the manuscript is suitable for publication.

GUDCA and TUDCA promotion of CRC was only done in the setting of GBx with supraphysiological addition of these bile acids. The question of whether or not physiological levels of these bile acids induced by GBx are themselves pro-tumorigenic remains unaddressed. Did the authors test if TUDCA or GUDCA addition in the absence of GBx can recapitulate GBx promotion of CRC? If so, this should be shown and discussed. Regardless, the authors should measure fecal levels of GUDCA and TUDCA after oral gavage for the experimental conditions they do show to understand how these relate to physiological levels induced by GBx.

While the experiments using riboflavin to inhibit BSH in B. breve and UDCA to inhibit 7β-HSDH in R. gnavus are supportive of the concept that bacterial modulation of TUDCA levels underlie tumorigenic potential, these inhibitors can have pharmacologic impacts beyond modulation of bile acids. Ideally, the authors should measure fecal TUDCA levels in the presence of these inhibitors to support the validity of their mechanisms of action. If this cannot be done, additional confounding actions of riboflavin and UDCA should at least be discussed in the manuscript.

While the authors have demonstrated decreased interaction between FXR and β-catenin, it remains unclear if this is the result of decreased FXR expression or some other mechanism. Indeed, the FXR co-IP results seem to show dramatically reduced β-catenin interactions in GBx +AD conditions compared to Sham + AD despite similar pull down of FXR. This suggests that decreased β-catenin interaction with FXR is due to some alternative mechanism, such as conditional interactions dependent on FXR binding to agonistic vs. antagonistic bile acids, however, this was neither tested nor discussed in the manuscript.

In the results section of the main text under the heading “B. breve and R. gnavus control colorectal tumorigenesis through

GUDCA production, the authors switch between describing GUDCA in human and TUDCA in mouse without explaining how these two different bile acids relate to each other. This is covered briefly in the discussion section but needs to appear in the results section as part of the logic flow so that the average reader can understand why the authors are presenting the data as it is.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Several issues have been clarified but a number of concerns remain.
The multiplicity of data and the fact that several figures are still not self-explanatory as mentioned in my previous review, make the presentation confusing.
Together with shortcomings, they make the conclusions uncertain.

1. The authors should make clear from the beginning (including the abstract) that the main findings were obtained in two mouse models of CRC. In the setting of these models, cholecystectomy appears to be an aggravating factor of CRC development, and information is lacking regarding dysbiosis and changes in BA metabolism at baseline in these models. These models are very different from patients whose intestine presumably was healthy.

2. Figure 5 is an example of figure, in which the multiplicity of conditions makes it confusing. It raises a major inconsistency : Cholecystectomy (GBx) + R. Gnavus + UDCA causes less CRC than cholecystectomy (h), whereas UDCA gavage should increase GUDCA, and therefor increases CRC (j). The level of TUDCA in UDCA-fed mice was not checked. Typically this figure is not self-explanatory: Abbreviations such as AD, BSH…are not defined; b, c it should be specified if this is fecal BAs; f there is an error in the legend R.gnauvs instead of R. gnavus.

3. Figure 7 is another example of not self-explanatory. Even though the authors propose a mechanism of how GUDCA and TUDCA modify the interaction between FXR and -catenin, it is not understandable why cholecystectomy has such a dramatic effect on FXR protein expression (Fig. 7e). Likewise, it is difficult to understand why OCA, which is an FXR activator, would have such a dramatic effect on FXR expression (Fig. 7e). Moreover, is not specified either in the Fig. legend or Methods, which part of the intestine was analyzed in Fig. 7e. Was it the ileum or the colon? Was it in tumoral or non-tumoral tissue? FXR is down-regulated in tumoral tissue, as a result of dedifferenciation (not to mistake for a direct effect of BAs).

4. The shift between the 2 models of CRC APCmin/+ vs AOM/DSS is not justified and also difficult to follow.

5. Key informations regarding Materials and methods, which were previously requested, such as which part of the intestine was analyzed cannot be provided only as supplementary especially as so little details are provided in Fig. legends.

6. The abstract is also poorly informative regarding the methods used.

Additional comments
7. Table S1 : change health for healthy; NAFLD for MAFLD (to define)
The authors wrote: “Importantly, previous studies showed that the occurrence rate of CRC increased markedly after cholecystectomy (Br J Cancer. 2003; 88: 79-83; PLoS One. 2017; 12: e0181852; Front Med. 2022; 9: 1000563). Therefore, integrating the previous literature and our findings suggests that the risk of CRC occurrence increases after cholecystectomy.”
I would delete markedly and change the 2nd sentence, for “However, the difference we found in the incidence of CRC between cholecystectomized and healthy subjects was no significant, which could be explained by a small sample size.”

8. Fig. S16: a. please define all abbreviations; b. color error of one dot (orange instead of black) to correct in the GUDCA group; c. why the statistical test was different in b and c? e. please show dysplastic mucosae and carcinoma by different arrows.

9. Fig. 4. The legends of g. and h. are not consistent

10. There are still a number of syntax errors, e.g. lines 82-83, “significantly difference”, lines 162-163, “To deeply explore… We…”; lines 256-257, “As conjugated…We previously…”; line 285, “…inhibitors UDCA…”

Reviewer #3

(Remarks to the Author)
The authors have performed key experiments that adequately address this reviewer’s concerns. The manuscript is now

suitable for publication.

Version 3:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors carefully addressed my comments and clarifications have been provided.
Yet, the conclusions are overstated. Colorectal carcinogenesis was exacerbated or accelerated in the mouse models rather than promoted as indicated in the results and in the title. Cholecystectomy exacerbates or accelerates colorectal carcinogenesis would be more appropriate. The mechanisms also remain questionable. Figure 8 indicates that the effect of cholecystectomy on colorectal carcinogenesis is mediated by GUDCA but GUDCA was not increased in cholecystectomized mice. It was increased in cholecystectomized humans who did not develop significantly more colorectal cancer.

Minor comment: In the discussion, lines 400-401 “Given that the AOM/DSS model places great emphasis on inflammation-related carcinogenesis, this model was used for the following study”. What does Following refers to?

Reviewer #1 (Remarks to the Author):

In this manuscript, the authors report changes in gut microbiota and in bile acid metabolism following cholecystectomy, both in patients and mice. They show that cholecystectomy increases colorectal carcinogenesis (CRC) in the azoxymethane (AOM) and dextran sodium sulfate (DSS) mouse model, and that this effect is reproduced by some of the changes in gut microbiota, i.e. a decrease in Bifidobacterium breve or an increase in Ruminococcus gnavus as observed in mice, and by an increase in glycol-ursodeoxycholic acid (GUDCA), as observed in humans. They attribute this effect to an inhibition of FXR and its interaction with β-catenin, which is reversed by obeticholic acid. Despite its interest, the work raises important concerns.

1. The causal relationship between cholecystectomy and colorectal cancer (CRC) or other complications in humans is still debated, whereas it is presented as well established (lines 346-351).

Response: We express our sincere gratitude for the invaluable constructive comments provided by the esteemed reviewers. Cholecystectomy is the most common procedure in biliary tract surgery, accompanied by long-term complications, such as recurrent choledocholithiasis and post-cholecystectomy syndrome ***(Am J Gastroenterol 2020; 115: 1191-1198.)*** Notably, previous meta-analysis showed that the risk of colon cancer after cholecystectomy was 30% higher ***(PLoS One 2017; 12: e0181852).*** In the current study, we observed that cholecystectomy is capable of promoting colorectal tumorigenesis in a manner dependent on the gut microbiota.

2. The information regarding the patients provided in Table S1, is very limited so that there could be many confounding factors notably metabolic factors, which could explain the differences in gut microbiota and bile acid profile between the two groups, besides cholecystectomy. The time elapsed since cholecystectomy apparently ranges between 1 and 20 years (lines 462-463), which makes the study group very heterogeneous, considering the adaptation of bile acids metabolism which may occur in patients post-cholecystectomy. Two patients in the group of cholecystectomy developed CRC, whereas it is not specified if CRC occurred before or after cholecystectomy, and how long after if this the case. In addition, the statistic method used to analyze patients' date should appear in the legend of Table S1.

Response: We have included the demographic data of both cholecystectomy patients and healthy controls in Supplementary Table 1, and subsequently conducted an analysis to identify any discernible differences between the two groups. And we also added the statistic method in the legend of Table S1.

3. The mechanism whereby cholecystectomy promotes CRC in mice was investigated by performing GUDCA gavage, whereas it is TUDCA which increases after cholecystectomy in mice. Likewise, whereas B. breve is decreased following cholecystectomy in mice, it seems to be increased in humans (Fig. 1C). Generally, figure legends are not self-explanatory, which makes the interpretation of data difficult.

Response: We greatly appreciate the valuable and constructive comment provided. In response to this, we have conducted additional experiments using TUDCA in a mouse model. The results of these experiments are presented in Fig. 5J-K and Fig. S16C-D, as provided in the attached document. Our findings indicate that TUDCA treatment significantly enhances the development of GBx-induced colorectal tumors (Fig. 5J and Fig. S16C), as well as tumor pathology (Fig. 5K and Fig. S16D). These results suggest that TUDCA may also play a role in promoting colorectal tumorigenesis. Based on previous studies and our own data, we posit that TUDCA and GUDCA promote GBx-mediated colorectal tumorigenesis.

We have diligently incorporated these new findings into the revised manuscript, specifically on Page 14.

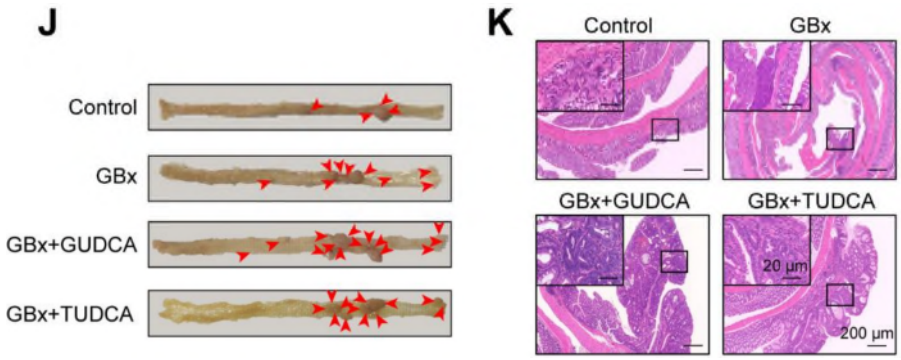


Fig. 5. *B. breve*- and *R. gnavus*-mediated bile acid metabolism is involved in colorectal tumorigenesis. (J-K) Representative images of mouse colon (J) and H&E staining (K) of mice treated with GUDCA or TUDCA following AOM/DSS model.

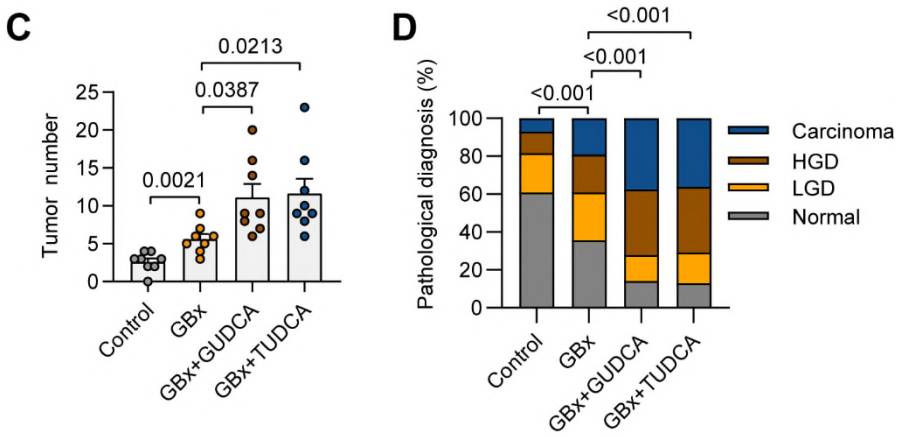


Fig. S16. Tumor number and quantitative analysis of H&E staining in mice, related to Fig. 5. (C) Average tumor number in mice treated with GUDCA or TUDCA following AOM/DSS model. (D) Quantitative analysis of H&E staining in each group.

In our study, we observed a significant decrease in the abundance of *B. breve* (Fig. 1D) and a notable increase in the abundance of *R. gnavus* (Fig. S4G) in the GBx population. we then conducted an analysis to determine the levels of *B. breve* and *R. gnavus* in mouse models following GBx. Our findings revealed an increase in the abundance of *R. gnavus* in the GBx+AOM/DSS group (Fig. S4F), while the abundance of *B. breve* showed a decrease (Fig. S4H). we also observed a significant decrease in the abundance of *B. breve* (Fig. S4I) and a notable increase in the abundance of *R. gnavus* (Fig. S4J) in the GBx group. Additionally, we observed that APC-GBx mice exhibited lower levels of *B. breve* (Fig. S4K) and higher levels of *R. gnavus* (Fig. S4L) compared to the APC-Sham group. Please refer to the attached document for detailed data on this matter.

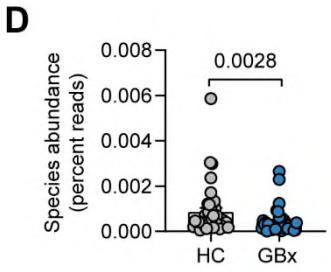


Fig. 1. The gut microbiota and bile acid profiles in cholecystectomy patients. (D) Relative abundance (percent reads) of *B. breve* based on metagenomics sequencing.

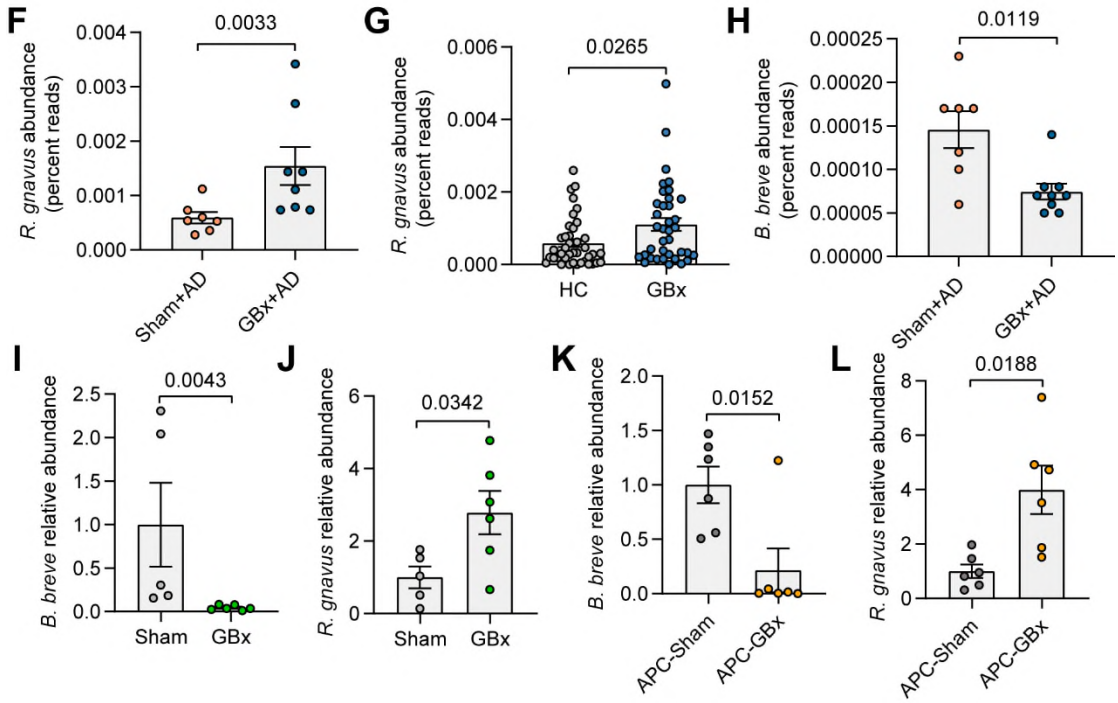
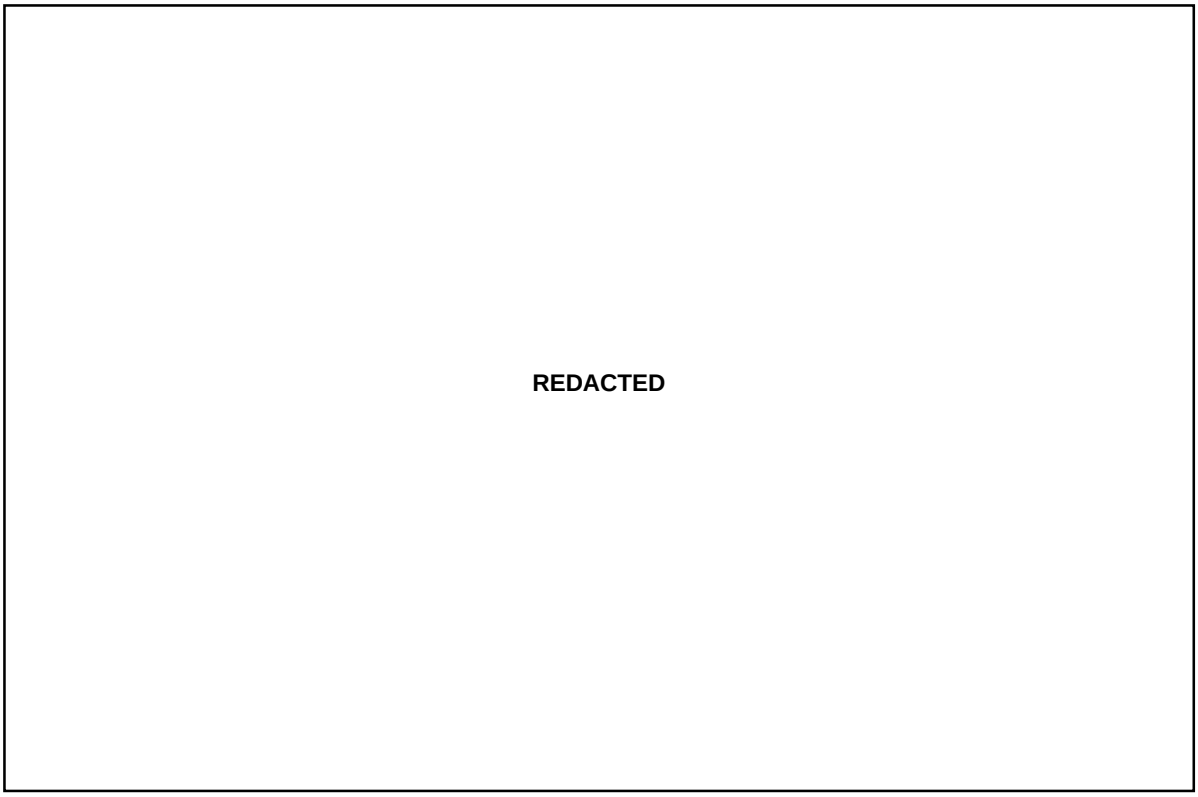


Fig. S4. The gut microbiota profile in Sham and GBx mice with or without AOM-DSS or APC genetic manipulation. (F) Relative abundance of *R. gnavus* between Sham+AOM/DSS and GBx+AOM/DSS mice. (G) The relative abundance of *R. gnavus* between healthy and cholecystectomy patients. (H) The relative abundance of *B. breve* between the Sham+AD and GBx+AD group. (I) qPCR analysis of *B. breve* abundance in Sham and GBx mice. (J) qPCR analysis of *R.gnavus* in Sham and GBx mice. (K) qPCR analysis of *B. breve* abundance between APC-Sham and APC-GBx mice. (L) qPCR analysis of *R. gnavus* in APC min/+mice.

To prevent any potential misunderstandings, we corrected the description in both the figure and figure legends, and detailed descriptions of the statistical analysis methods have also been incorporated into the respective figure legends. The updated version of the figure and its corresponding legends are provided in the attached document.

4. The English syntax needs to be thoroughly revised in particular to express quantitative data properly, e.g. “more severe tumor distribution” (lines 355-356) does not mean anything. The meaning of several other sentences is unclear, for example, lines 133 and 147: “tumor distribution…larger and wider”; line 147 “colorectal tumor distribution…greater ; line 200 more obvious CRC phenotype; line 393 “a major role in this process”: which process does this refer to?; lines 396-397, “the transformation from CDCA to UDCA …has proven feasible”; lines 432-433 “Our results showed that the combination….to the control group”. Line 432, reference 33 is not appropriate.

Response: We express our sincere gratitude for the invaluable constructive comments provided by the esteemed reviewers. The manuscript has been submitted to Springer Nature for professional language editing. Please find attached the corresponding certificate for your reference.



In the revised version, we have corrected the improper reference at line 432 ***(Acta Pharm Sin B 2024;14:1241-1256).***

5. The number of replicates is not indicated for all quantitative data in figure legends. Details are also lacking regarding the histopathological analysis of the colon in mice. The score was the average of scores determined by two experts. Information is lacking regarding the intra- and inter-observer variability in this scoring analysis? Information is also lacking regarding the distribution of lesions along the colon, i.e., proximal vs. distal. How were the different grades of histological severity found in each mouse take into account. In addition, it is not specified which part of the colon or if the entire colon were used for RT-PCR analyses.

Response: To ensure comprehensive reporting and research transparency, we have included the number of technical replicates in each figure legend. It is important to note that all data presented in this study are representative results derived from a minimum of three independent experiments.

The histologic injury scores (0-12) were evaluated in a blinded manner by a gastrointestinal pathologist based on the degree of epithelial damage and inflammatory infiltrate in the mucosa, submucosa and muscularis/serosa ***(J Clin Invest 2005;115:695-702).*** And intestinal tumors were assessed by an experienced pathologist, according to the pathology of mouse models of intestinal cancer: consensus report and recommendations ***(Gastroenterology 2003;134:762-77; Gastroenterology***

2013;144:705-17). And the content of review mainly includes: the diameter and number of dysplasia/neoplasia/ tumors, the depth of tumor invasion, the area affected by inflammation. Lesions of low (LGD) and high-grade dysplasia (HGD) and adenocarcinoma were graded and counted on each section. Moreover, these colonic lesions are located in the distal colon.

It is widely known that there exist individual differences among mice, and mice within the same group may exhibit different levels of histological severity. After obtaining the histological score data of mice, we carried out the analysis strictly in accordance with the statistical analysis method. And to ensure the accuracy and appropriateness of our statistical analysis, we sought the expertise of a specialized statistician who carefully reviewed and rechecked all the data analysis. The specific details of the statistical analysis methods have been added to each figure legend.

Furthermore, we conducted PCR analysis utilizing colon cancerous tissues to delve deeper into the specific mechanism underlying GBx-induced tumorigenesis, building upon previous reports **(*Gut 2023; 72(4): 710-721; Mol Cancer 2020; 19(1):46; Int J Biol Sci. 2023; 19 (14):4393-4410*).**

6. Some statements like “after cholecysctectomy, the enterohepatic circulation of bile acid was accelerated…” (line 115) was not shown in the present study, and if this is the case, the proportion of conjugated bile acids should be decreased rather than increased as found here (line 118).

Response: We express our sincere appreciation for the valuable comment provided. In the revised manuscript, we have rectified these improper expressions.

7. All abbreviations should be defined.

Response: In the revised manuscript, we have defined all the abbreviations.

Reviewer #2 (Remarks to the Author):

This study by Tang B. and colleagues is of great interest for understanding the contribution of cholecystectomy to CRC initiation and progression. It also reveals potential drug targets in this specific scenario of CRC initiation. Most of the experiments are well designed and executed. This is a study of interest for the field of CRC. The experiments, figures and text are well designed and clear. However, there are many relevant aspects that should be significantly improved before its publication in Nature Communications.

MAJOR COMMENTS:

General advice:

The number of mice (n) used in each experiment should be indicated in all figures.

Response: To ensure comprehensive reporting and research transparency, we provided annotations for the number of mice in each group for each experiment in the figure legend in the revised manuscript.

1. Cholecystectomy and intestinal proliferation:

Humans develop tumors in colon or rectum but rarely in small intestine.It is necessary to quantify with greater detail the proliferation of normal mucosa in control and GBx mouse intestines. Several questions should be answered:

1.1. GBx does affect only the proliferation of small intestinal cells?

1.2. Is it also affecting the proliferation of colon or rectum mucosa?

1.3. Which are the cells with increased proliferation? Enterocytes, stem cells, other lineages? This characterization will help to understand whether an abnormal increased proliferation rate could be required for tumor initiation from a particular cell of origin?

Response: We deeply appreciate the valuable and constructive comment provided. In our study, we concentrated on the impacts of cholecystectomy on colorectal tumorigenesis. Our results demonstrated that in both the AOM-DSS and APC$^{min/+}$ mouse models, the number of ki-67 positive cells in colorectal tumors increased after cholecystectomy (Fig. 2H and Fig. S2F), suggesting that cholecystectomy promotes the proliferation of colorectal tumors.

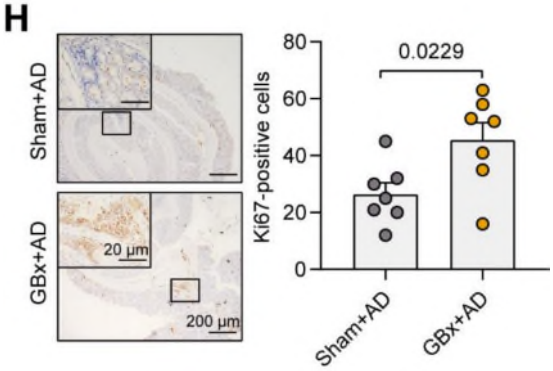


Fig. 2. Cholecystectomy promotes colorectal tumorigenesis in C57BL/6 mice. (H) Representative images and semiquantitative analysis of Ki67 staining of mouse colons.

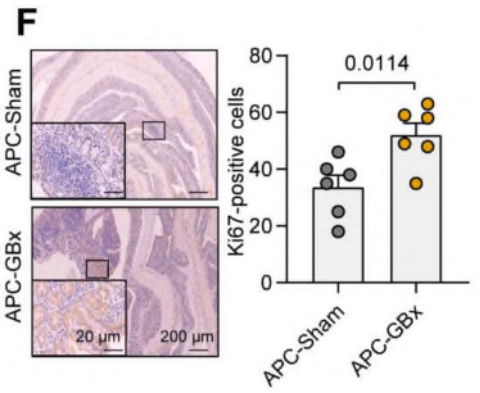


Fig. S2. Cholecystectomy facilitates colorectal tumorigenesis in APCmin/+ mice. (F) Representative images of Ki67 staining of mouse colons and semiquantitative analysis.

Previous studies have reported that antagonism of intestinal FXR induces proliferation and DNA damage in Lgr5+ cancer stem cells ***(Cell 2019; 176: 1098-1112.e18).*** And intestinal organoids are three-dimensional tissue analogues formed by crypts or single stem cells from the intestine in a culture system in vitro, with self-renewal and differentiation capabilities ***(Cell Stem Cell. 2018; 23: 787-793.e6).*** In our manuscript, we utilized an intestinal epithelial organoid model from GBx+AOM/DSS mice to explore the specific mechanism. Remarkably, GUDCA inhibited the growth of organoids induced by GUDCA (Fig. 7H), indicating that cholecystectomy promotes the proliferation of cancer stem cells.

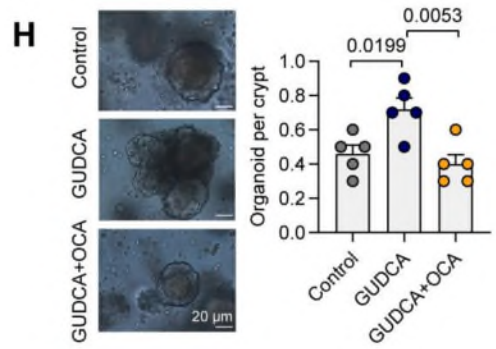


Fig. 7. OCA prevents cholecystectomy-induced colorectal tumorigenesis. (H) Representative image and quantification of organoids generated from GBx+AOM/DSS mice treated with control, GUDCA, or GUDCA+OCA.

2. Intestinal permeability:

GBx mice present an increased intestinal permeability. Several questions arise:

2.1. Is it due to a defective cell differentiation in mucosa?

2.2. Is it due to abnormal mucosa absorption physiology?

2.3. Is it due to a defective barrier/cell-to-cell contact monolayer epithelia?

Response: We deeply appreciate the valuable and constructive comment provided. We have conducted additional experiments and analyses comparing the sham and GBx

groups in the absence of AOM-DSS or APC genetic manipulation. Our findings revealed the intestinal barrier function of GBx mice was slightly impaired without significantly difference (Fig. S1C). However, we observed a notable decrease in the expression of barrier-related molecules, namely ZO-1 (Fig. S1D) and Occludin (Fig. S1E) in the GBx group, suggesting that cholecystectomy gives rise to defective barrier/cell-to-cell contact monolayer epithelia and increases intestinal permeability.

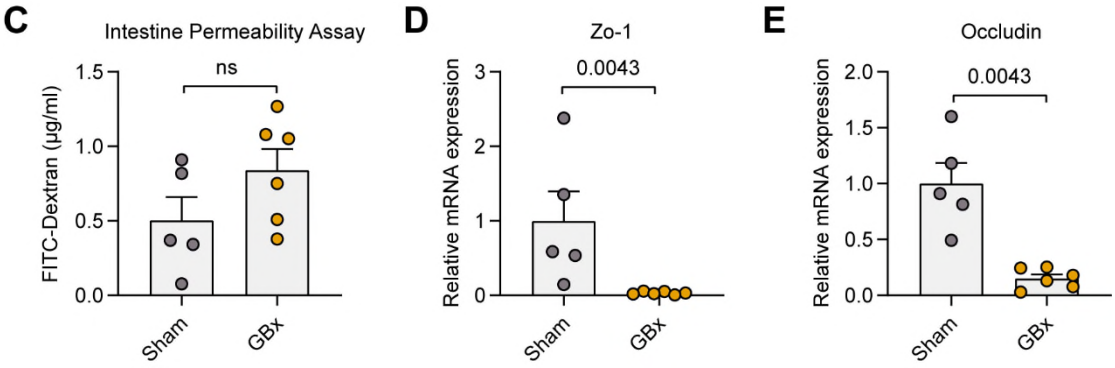


Fig. S1. Cholecystectomy induced changes of gut barrier function and epithelial integrity in mice. (C) Intestinal permeability measured by FITC-dextran in mice after operation. (D-E) Relative expression of Zo-1 (D) and Occludin (E) in Sham and GBx mice.

3. Effect of bile acid on bacteria:

B. breve and R. gnavus are both increased in patients with GBx? A quantification of B. breve should be shown. How is possible that B.breve is sensitive and R. gnavus is resistant to Bile acids?

Response: In our study, we observed a significant decrease in the abundance of *B. breve* (Fig. 1D) and a notable increase in the abundance of *R. gnavus* (Fig. S4G) in the GBx population. We then conducted an analysis to determine the levels of *B. breve* and *R. gnavus* in mouse models following GBx. Our findings revealed an increase in the abundance of *R. gnavus* in the GBx+AOM/DSS group (Fig. S4F), while the abundance of *B. breve* showed a decrease (Fig. S4H). we also observed a significant decrease in the abundance of *B. breve* (Fig. S4I) and a notable increase in the abundance of *R. gnavus* (Fig. S4J) in the GBx group. Additionally, we observed that APC-GBx mice exhibited lower levels of *B. breve* (Fig. S4K) and higher levels of *R. gnavus* (Fig. S4L) compared to the APC-Sham group. Please refer to the attached document for detailed data on this matter.

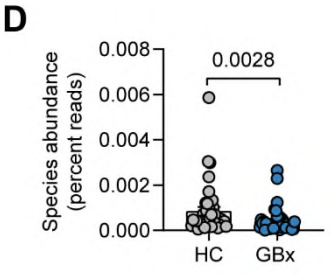


Fig. 1. The gut microbiota and bile acid profiles in cholecystectomy patients. (D) Relative abundance (percent reads) of *B. breve* based on metagenomics sequencing.

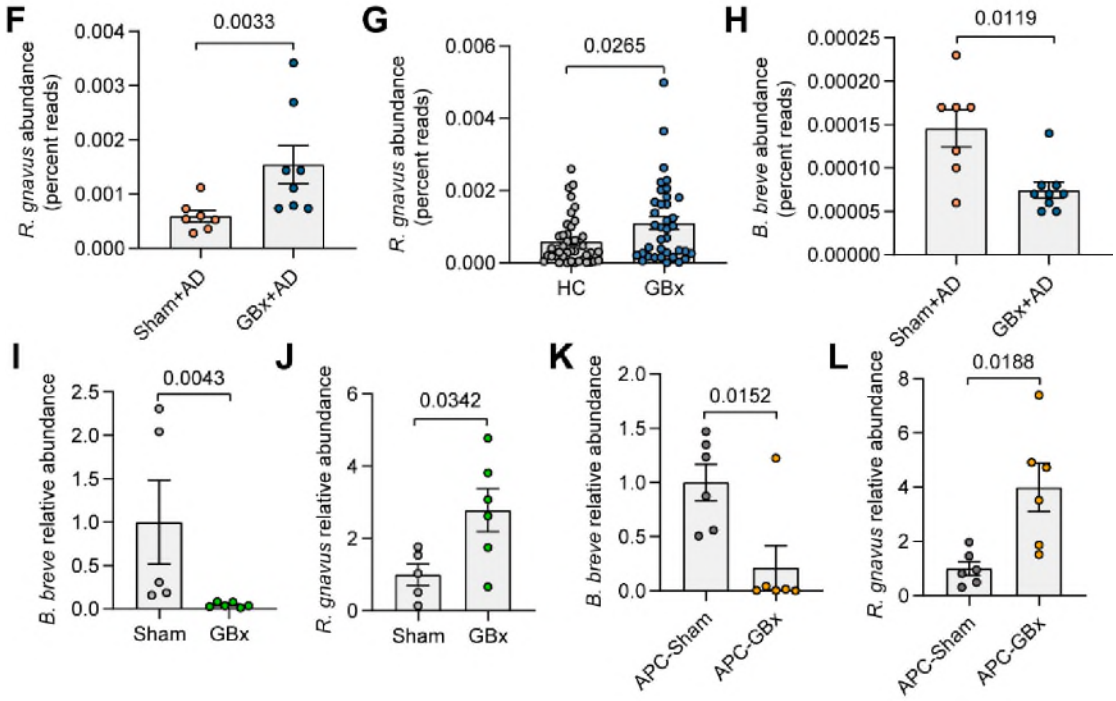


Fig. S4. The gut microbiota profile in Sham and GBx mice with or without AOM-DSS or APC genetic manipulation. (F) Relative abundance of *R. gnavus* between Sham+AOM/DSS and GBx+AOM/DSS mice. (G) The relative abundance of *R. gnavus* between healthy and cholecystectomy patients. (H) The relative abundance of *B. breve* between the Sham+AD and GBx+AD group. (I) qPCR analysis of *B. breve* abundance in Sham and GBx mice. (J) qPCR analysis of *R.gnavus* in Sham and GBx mice. (K) qPCR analysis of *B. breve* abundance between APC-Sham and APC-GBx mice. (L) qPCR analysis of *R. gnavus* in APC min/+mice.

Bile tolerance is one of the most crucial characteristics of gut bacteria since it determines their capacity to survive in the intestine and consequently their ability to perform their functions. Apart from their normal physiological functions, bile acids are extremely toxic to microorganisms that are not adapted to intestinal conditions. Bile salt can alter cell membranes and cause DNA damage, thereby exerting antibacterial effects ***(Front Microbiol. 2013; 4:396).*** In our manuscript, we observed the growth of B. breve was inhibited in bile salts in a concentration-dependent manner (Fig. S14A), suggesting *B. breve* is sensitive to bile acid.

To be able to survive in gastrointestinal transportation and temporarily colonize our intestines, theses bacteria must evolve specific defense mechanisms to overcome the harmful effects of bile salts. These adaptations are composed of the efflux of bile salts from cells, the hydrolysis of bile salts, and the structural and compositional alterations of bacterial cell membranes ***(Front Microbiol. 2013; 4:396; Biotechnol Adv. 2018; 36(3): 682-690).*** In our manuscript, our results indicated that *R. gnavus* was resistant to bile salts (Fig. S14B).

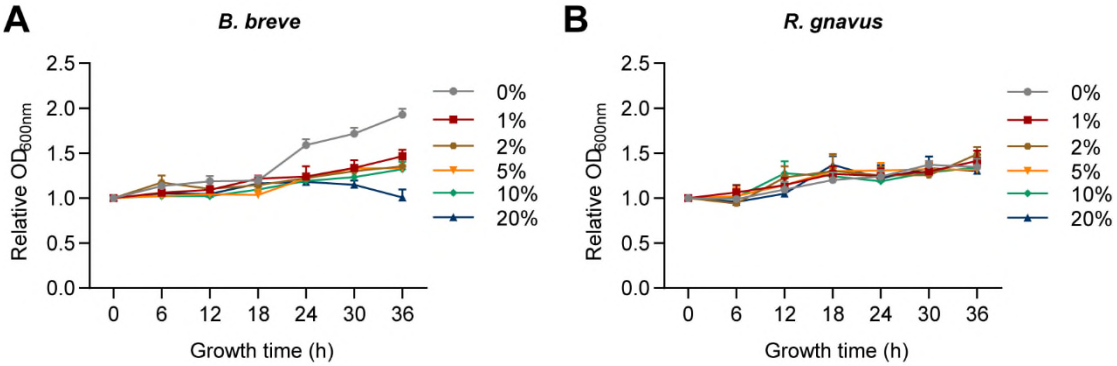


Fig S14. Bile acid salts alter the growth of B. breve and R. gnavus, related to Fig. 5. (A) Growth curve of B. breve after treatment with different concentrations (wt/vol) of bile acid salts. (B) Growth curve of R. gnavus after treatment with different concentrations (wt/vol) of bile acid salts.

4. Fecal Microbiota Transplantation:

Authors should dissect the contribution of microbiota versus non-microbiota components from donor fecal material when transplanted into mice. For instance, GBx fecal material from patients or mice present a different bile acid composition. Does it affect tumorigenesis in transplanted mice?

Response: We greatly appreciate the valuable and constructive comment provided. We proceeded to conduct humanized and murine-derived fecal microbiota transplantation (FMT) experiments in order to assess the influence of the microbiota from individuals who underwent cholecystectomy and corresponding control groups on colorectal tumorigenesis (Fig. 4A-F, Fig. S6, and Fig. S7). Subsequently, we assessed the role of *B. breve* and *R. gnavus* in our model through a single bacterium gavage experiment (Fig. S9). These results assessed the contribution of microbiota components from donor fecal material in tumorigenesis in transplanted mice.

We previously discovered that GUDCA was elevated in the population after GBx, and TUDCA increased in GBx mice. Consequently, we carried out additional experiments using GUDCA and TUDCA in a mouse model. Our findings indicate that both GUDCA and TUDCA treatment significantly enhances the development of GBx-induced colorectal tumors (Fig. 5J and Fig. S16C), as well as tumor pathology (Fig. 5K and Fig. S16D). These results assessed the contribution of non-microbiota components from donor fecal material in tumorigenesis in transplanted mice.

Furthermore, we conducted a series of experiments using the microbiota and bile salt hydrolase (BSH) inhibitor riboflavin or 7β-hydroxysteroid dehydrogenase (7β-HSDH) inhibitor UDCA to elucidate the specific mechanisms of the microbiota and bile acids (non-microbiota components) in our model (Figure 5H-I and Figure S16A-B).

Taken together, these results dissect and clarify the contribution of microbiota and non-microbiota components from donor fecal material when transplanted into mice.

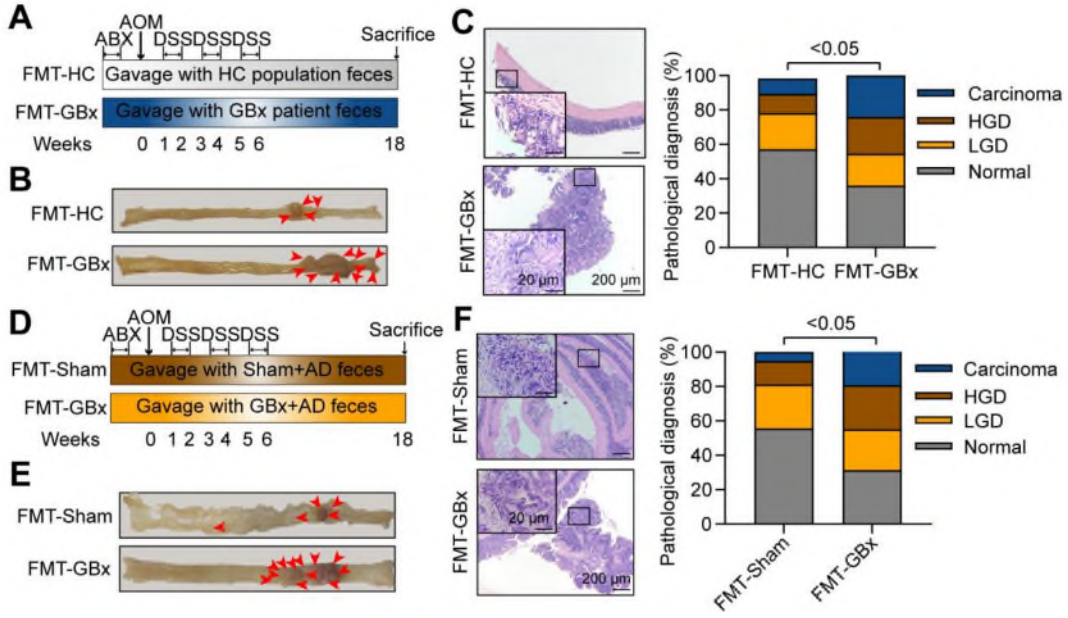


Fig. 4. Cholecystectomy promotes colorectal tumorigenesis in a gut microbiota-dependent manner. (A) Experimental design for patient FMT mouse model. (B) Representative images of mouse colons. (C) Representative images of H&E staining and semiquantitative analysis. (D) Schematic overview of mouse fecal FMT model. (E) Representative images of the colon. (F) Representative images of H&E staining and semiquantitative analysis.

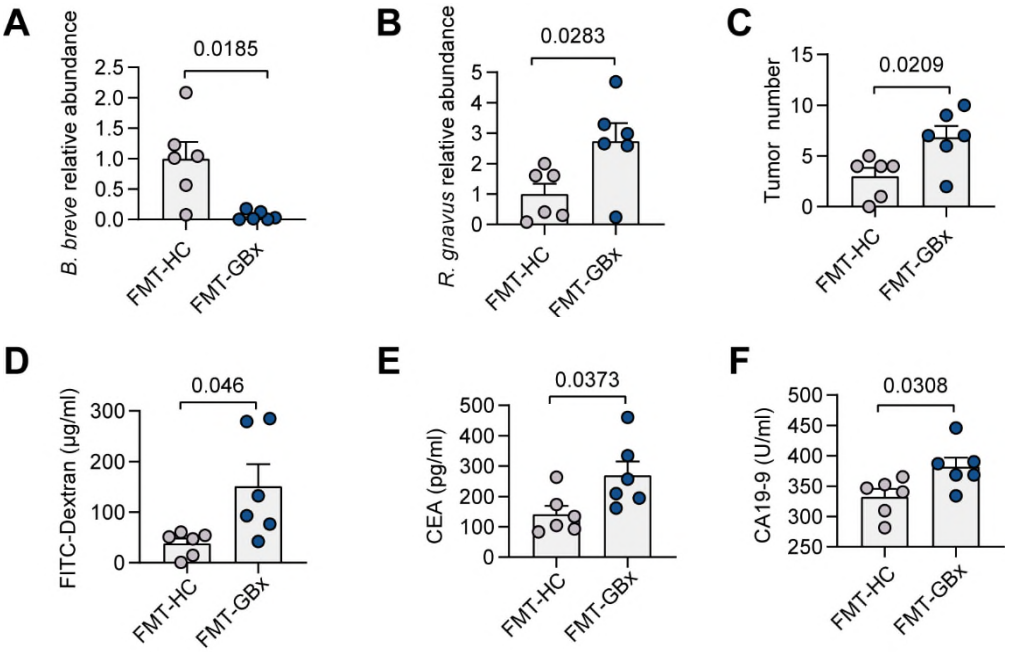


Fig. S6. FMT derived from GBx patients promotes colorectal tumorigenesis, related to Fig. 4. (A) qPCR

analysis of *B. breve* abundance after FMT experiments. (B) qPCR analysis of *R. gnavus* abundance between FMT-HC and FMT-GBx group. (C) Average tumor number of the FMT recipient mice. (D) Intestinal permeability measured by FITC-dextran. (E-F) Serum CEA (E) and CA19-9 (F) levels in FMT recipient mice.

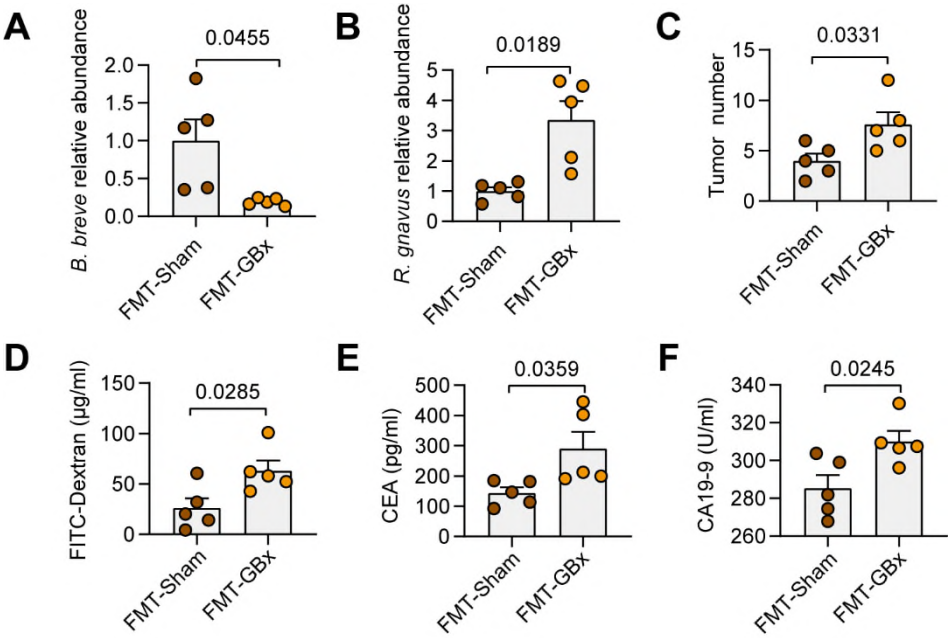


Fig. S7. FMT derived from tumor-bearing GBx mice promotes colorectal tumorigenesis, related to Fig. 4. (A-B) qPCR analysis of *B. breve* (A) and *R. gnavus* (B) abundance after mouse FMT experiments. (C) Average tumor number of the mouse FMT recipient mice. (D) Intestinal permeability measured by FITC-dextran. (E-F) Serum CEA (E) and CA19-9 (F) levels in mouse FMT recipient mice.

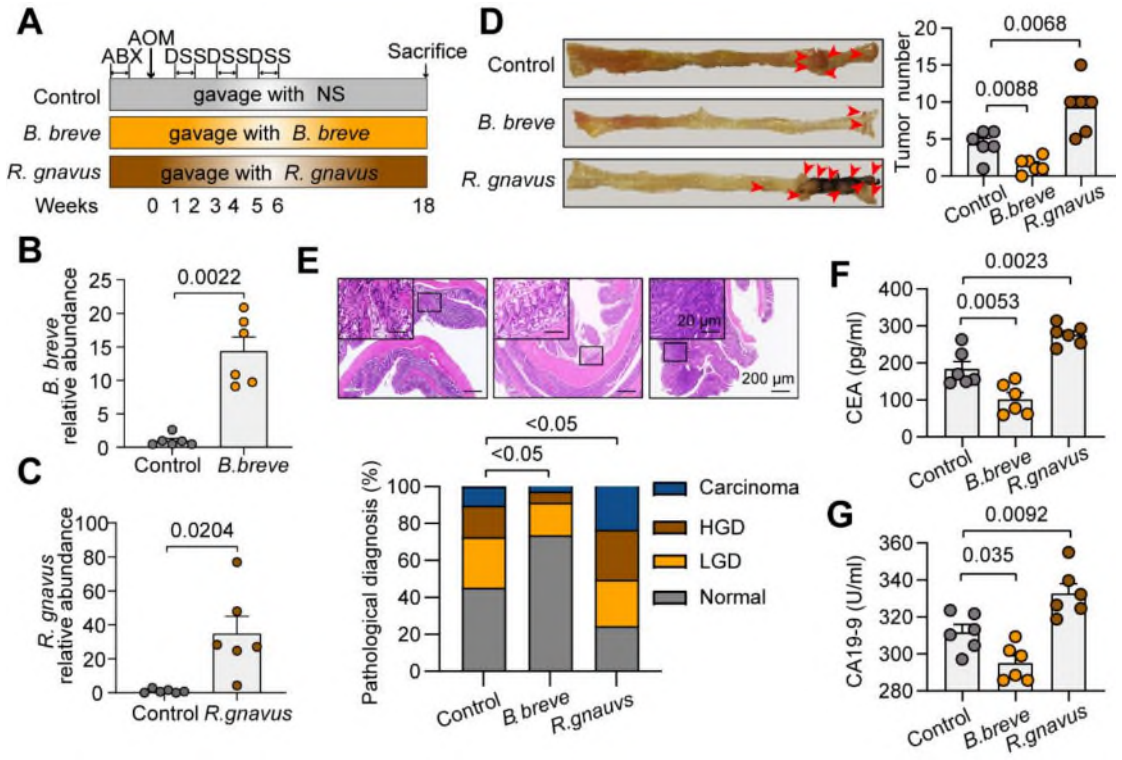


Fig. S9. *B. breve* gavage reduces colorectal tumorigenesis, while *R. gnavus* gavage promotes colorectal tumorigenesis, related to Fig. 5. (A) Experimental design for mice orally gavaged with NS (control), *B. breve* or *R. gnavus.* (B) qPCR analysis of *B. breve* abundance after *B. breve* gavage experiments. (C) qPCR analysis of *R. gnavus* abundance after *R. gnavus* gavage treatment. (D) Representative colonic morphologies and average tumor number in each group. (E) Representative images of H&E staining and semiquantitative analysis.

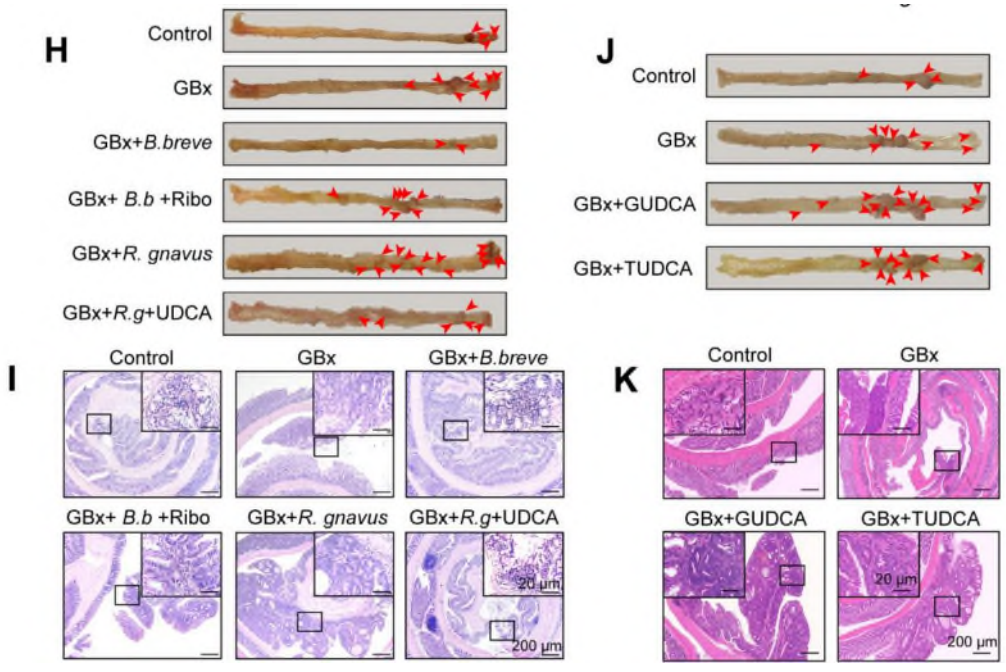


Fig. 5. *B. breve*- and *R. gnavus*-mediated bile acid metabolism is involved in colorectal tumorigenesis. (H-I) Representative images of mouse colon (H) and H&E staining (I) after *B. breve* or *R. gnavus* gavage, treated with

BSH inhibitors riboflavin or 7β-HSDH inhibitors UDCA, respectively. (J-K) Representative images of mouse colon (J) and H&E staining (K) of mice treated with GUDCA or TUDCA following AOM/DSS model.

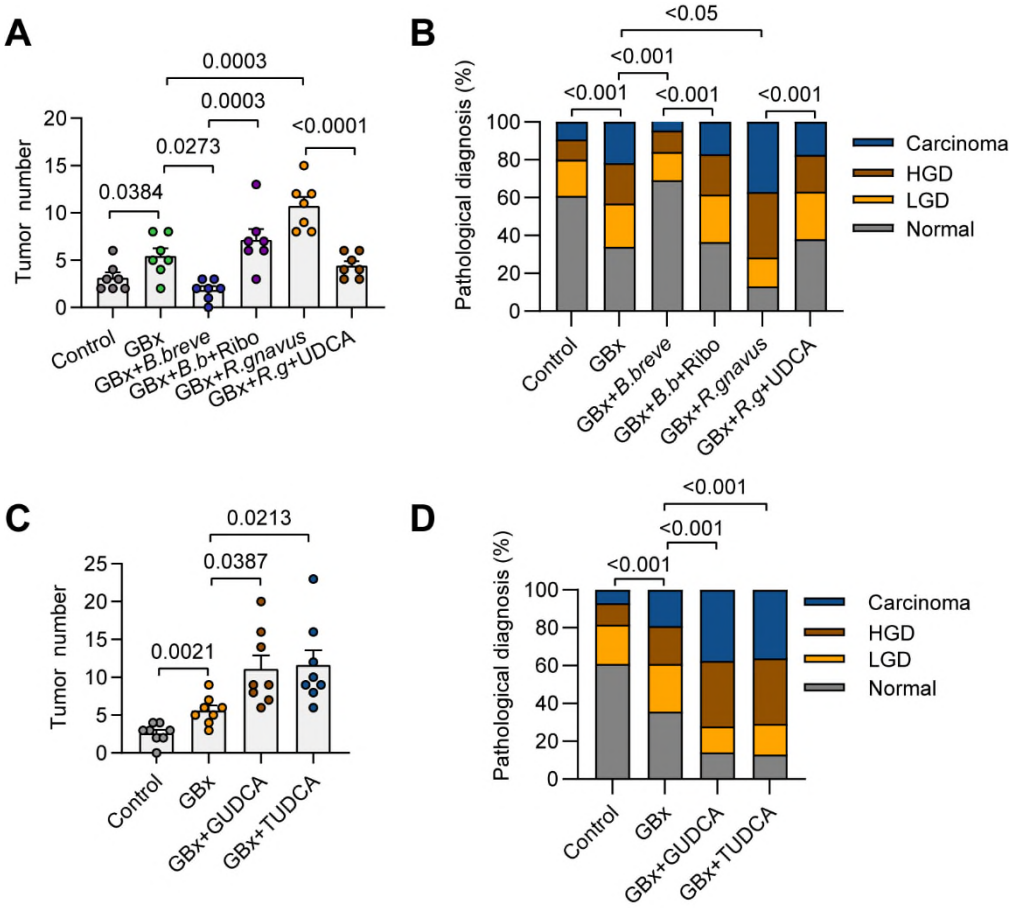


Fig. S16. Tumor number and quantitative analysis of H&E staining in mice, related to Fig. 5. (A) Average tumor number in mice after *B. breve* or *R. gnavus* gavage, treated with BSH inhibitors riboflavin or 7β-HSDH inhibitors UDCA, respectively. (B) Quantitative analysis of H&E staining in each group. (C) Average tumor number in mice treated with GUDCA or TUDCA following AOM/DSS model. (D) Quantitative analysis of H&E staining in each group.

5. Wnt pathway:

The activation of Wnt/beta-catenin pathway by GBx or GUDCA is not correctly measured. Wnt activation should be related to NUCLEAR beta-catenin and not total beta-catenin. This applies for western blot analyses or histoligies all along the study (Figure 5, 6 & corresponding supplementary). Most of beta-catenin is accumulated in cell-to-cell contacts in normal and tumor cells. The active pool is that accumulated in the nucleaus as transcriptional coactivator.

5.1. A reporter assay of Wnt/beta-catenin and PXR/beta-catenin transcriptional activity should be done. This means a reporter (luciferase) gene under the control of TCF or PXR response elements transfected in CRC cells treated with GUDCA.

5.2. Better images with magnification to observe nuclear beta-catenin should be shown.

5.3. An intervention proof-of-concept experiment with a Wnt inhibitor such a TNK inhibitor should be done in vivo to confirm is relevant in GBx induced tumorigenesis.

Response: In line with the reviewer's valuable suggestion, we have conducted additional experiments to further validate the interaction between FXR and β-catenin, as well as to elucidate the role of FXR in β-catenin downstream signaling. To examine the association of FXR with β-catenin, we performed co-immunoprecipitation (Co-IP) analysis using nuclear lysates. Our results revealed a decreased interaction between FXR and β-catenin in GBx mice (Fig. 6J). Conversely, we observed an increased interaction between β-catenin and TCF4 in GBx mice (Fig. 6K). These findings provide further support for the involvement of FXR in the regulation of β-catenin signaling.

Furthermore, to strengthen our understanding of the functional implications of FXR/β-catenin interaction in GBx-induced tumorigenesis, we incorporated additional assays into our study. We employed the TOP/FOP-Flash luciferase reporter assay to assess β-catenin transcriptional activity (Fig. S19D). The results indicated that FXR knockdown led to increased β-catenin transcriptional activity. Additionally, we performed cell viability analysis and found that FXR knockdown significantly enhanced cell viability, an effect that could be reversed by the Wnt signaling inhibitor XAV-939 (Fig. S19E).

We have incorporated these new findings into the revised manuscript on page 16.

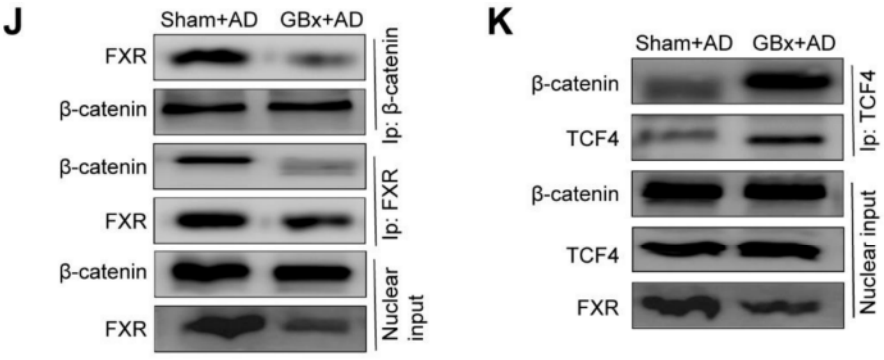


Fig. 6. FXR/β-catenin signaling is involved in cholecystectomy-related colorectal tumorigenesis. (J) Co-immunoprecipitation of the interaction between FXR and β-catenin. (K) Co-IP of the interaction between TCF4 and β-catenin.

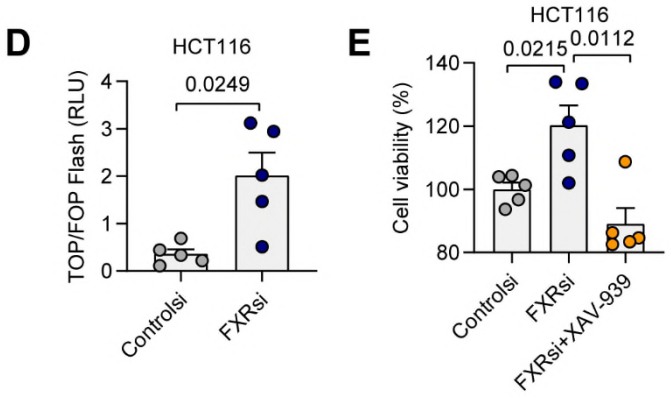


Fig. S19. FXR/β-catenin signaling is involved in colorectal tumorigenesis induced by cholecystectomy, related to Fig. 6. (D) The effect of FXR knockdown on the luciferase activity of TOP/FOP-Flash reporter plasmid in HCT116 cells. (E) The effect of FXR knockdown and XAV939 on the viability of colon cancer cells detected by CCK8 assays.

To enhance clarity, we have enlarged the aforementioned images to observe nuclear beta-catenin, which are now presented in Fig. 6H. For further details, please refer to the attached document.

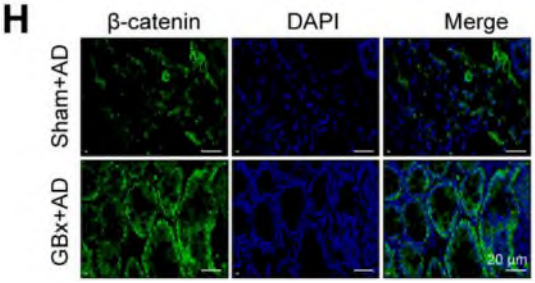


Fig. 6. FXR/β-catenin signaling is involved in cholecystectomy-related colorectal tumorigenesis. (H) Immunofluorescence analysis of β-catenin. β-Catenin, green; DAPI, blue.

6. OCA therapy:

Obeticholic acid (OCA) is a FXR agonist ligand. How it also induces the expression of FXR gene? Please explain. Again Wnt pathway activation should be precisely measured by nuclear and no total beta-catenin.

Response: We greatly appreciate the valuable and constructive comment provided. Previous research has indicated that OCA is capable of activating the FXR and modifying the expression of numerous FXR target genes ***(J Hepatol. 2016; 64(5): 1158-1166).*** Interestingly, there is evidence suggesting a direct effect of OCA on FXR expression, and on downstream FXR-activated pathways in organoids from $APC^{min/+}$ mice ***(Cell. 2019; 176(5): 1098-1112. e18)***. In our manuscript, we discovered that OCA treatment powerfully induced the expression of FXR target genes in both GBx mice (Fig. S20D-E) and organoids (Fig. S20G-H). These results suggested that OCA could induce the expression of FXR gene. (attached below)

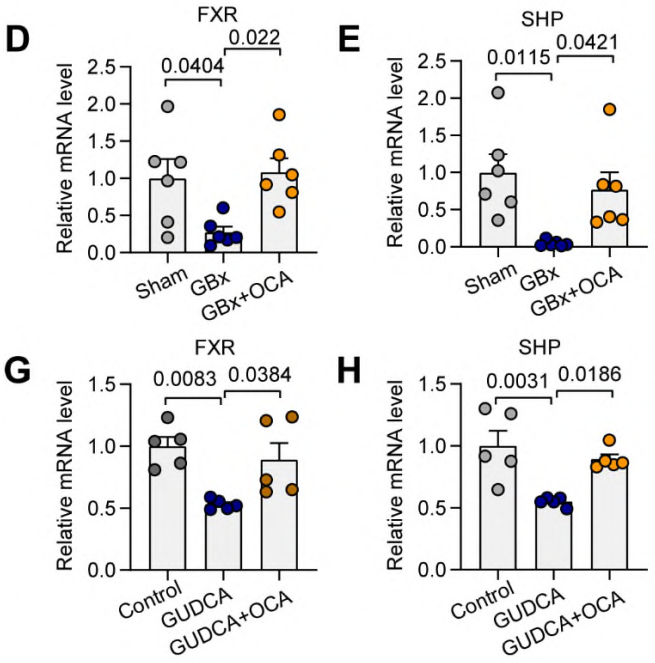


Fig. S20. FXR/β-catenin signaling is altered after OCA treatment in GBx mice or GUDCA-treated intestinal organoid, related to Fig.7. (D-E) Relative expression of FXR (D) and SHP (E) in each group. (G-H) Relative expression of FXR (G) and SHPin intestinal organoid treated with GUDCA and OCA.

The regulatory mechanisms of FXR gene expression mainly include: ligand activation, transcription factor regulation, and signal pathway-mediation, etc. ***(Biochim Biophys Acta. 2011; 1812(8): 842-50).*** Currently, the mechanism through which OCA directly induces the expression of FXR target genes remains incompletely understood. It is worth mentioning that structural analyses of the ligand-binding domain (LBD) of rat and human FXR bound to synthetic or natural ligands and coactivator peptides have provided significant insights into the mechanisms of FXR activation by its ligands ***(Mol Cell. 2003; 11(4): 1079-92; Mol Cell. 2003; 11(4): 1093-100).*** FXR is a nuclear receptor that awaits the binding of an appropriate ligand in the nucleus to exert its function. OCA has a good affinity for the LBD of FXR. Therefore, we speculate that after OCA binds to FXR, it will cause a conformational change in FXR. This conformational change leads to the release of bound corepressor proteins and recruitment of coactivator proteins to the DNA-binding site, thereby regulating the expression of FXR target genes. On the other hand, bile acids are the natural ligands of FXR. As a synthetic bile acid analogue, OCA is capable of regulating the intracellular signal network in a similar way. The addition of OCA changes the bile acid signal balance within cells and regulates the intracellular signal network. These altered signal molecules further regulate the expression of FXR target genes to maintain the homeostasis and metabolic physiological functions of bile acids.

Given that FXR is predominantly located in the nucleus, we utilized nuclear lysates for the assessment of FXR and β-catenin association. Specifically, we have observed a decrease in the association between FXR and β-catenin (Fig.6J), alongside an increase in the interaction between β-catenin and TCF4 in GBx mice (Fig. 6K). These discoveries provide stronger support for the involvement of FXR in the regulation of β-catenin signaling. We have diligently included the aforementioned new data in the revised manuscript, specifically on Page 16.

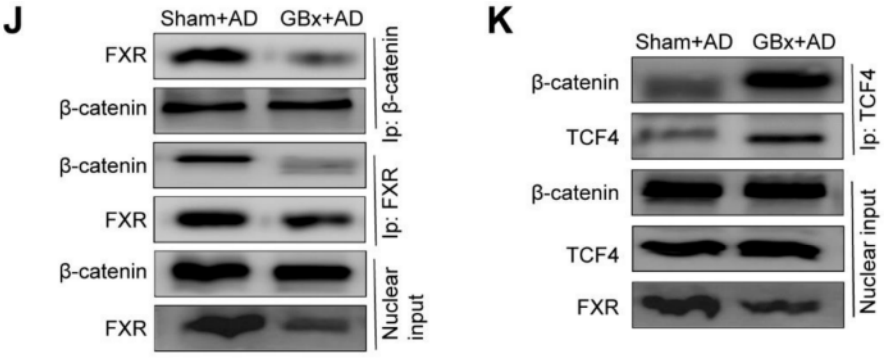


Fig. 6. FXR/β-catenin signaling is involved in cholecystectomy-related colorectal tumorigenesis. (J) Co-immunoprecipitation of the interaction between FXR and β-catenin. (K) Co-IP of the interaction between TCF4 and β-catenin.

7. FXR/beta-catenin axis:

A mutant version of FXR unable to bind beta-catenin should be used to prove that OCA is unable to induce Wnt target genes. On the contrary, part of the phenotypes indicated could be due to canonical TCF/beta-catenin pathway upon GBx/GUDCA signaling.

Response: We highly appreciate the valuable comment provided. To strengthen our understanding of the functional implications of FXR/β-catenin interaction in GBx-induced tumorigenesis, we incorporated additional assays into our study. We employed the TOP/FOP-Flash luciferase reporter assay to assess β-catenin transcriptional activity (Fig. S19D). The results indicated that FXR knockdown led to increased β-catenin transcriptional activity. Additionally, we performed cell viability analysis and found that FXR knockdown significantly enhanced cell viability, an effect that could be reversed by the Wnt signaling inhibitor XAV-939 (Fig. S19E).

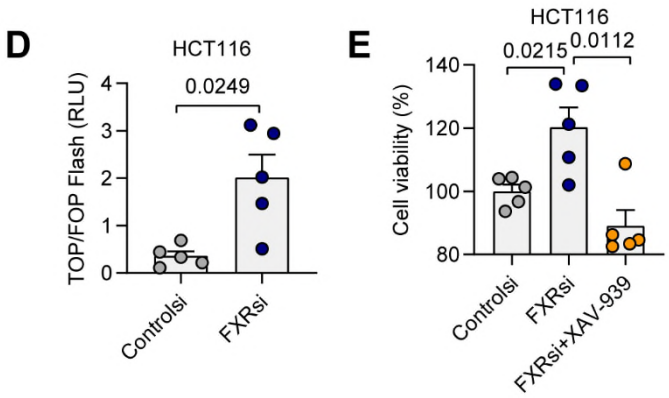


Fig. S19. FXR/β-catenin signaling is involved in colorectal tumorigenesis induced by cholecystectomy, related to Fig. 6. (D) The effect of FXR knockdown on the luciferase activity of TOP/FOP-Flash reporter plasmid in HCT116 cells. (E) The effect of FXR knockdown and XAV939 on the viability of colon cancer cells detected by CCK8 assays.

In addition, we observed that FXR and SHP expression decreased, β-catenin and c-Myc expression increased, binding of FXR and catenin decreased, while binding of β-catenin and TCF4 increased after GBx, which can be reversed by OCA (Fig. 7E-G, Fig. S20D-F). We then used an intestinal epithelial organoid model from GBx+AOM/DSS mice to examine the effects of OCA. Notably, OCA inhibited GUDCA-induced organoid growth (Fig. 7H). Mechanistically, we found that OCA could induce FXR and SHP (Fig. S20G-H) expression, and down-regulate Myc expression (Fig. S20I).

These results collectively validate the role of FXR/catenin in linking GBx to colorectal tumorigenesis. And we have diligently included the aforementioned new data in the revised manuscript, specifically on Page 17.

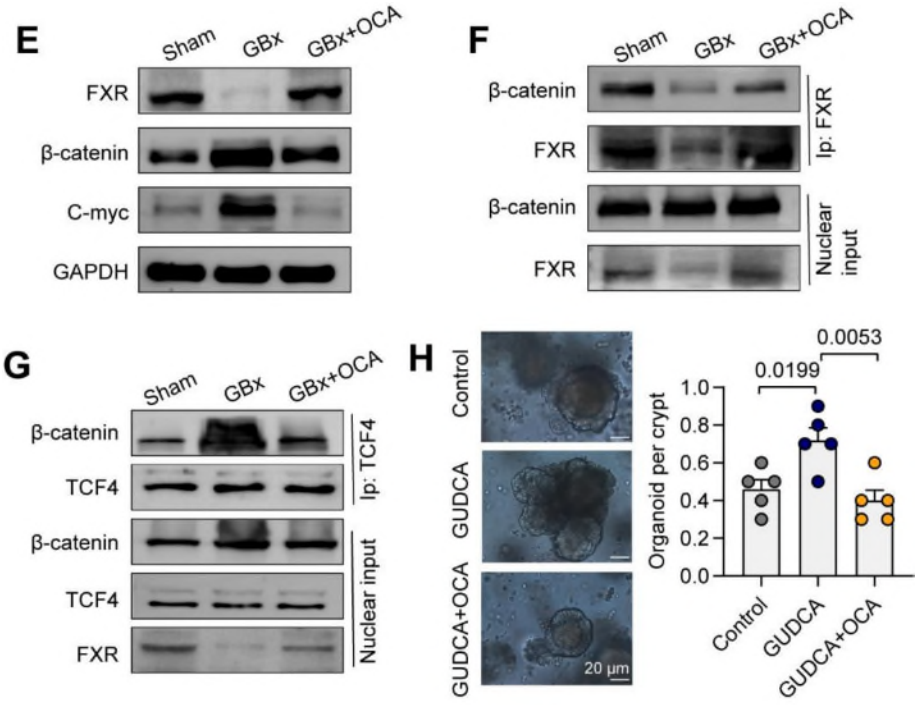


Fig. 7. OCA prevents cholecystectomy-induced colorectal tumorigenesis. (E) Protein expression of FXR, β-catenin, C-myc, and GAPDH in each group. (F) Co-immunoprecipitation of the interaction between FXR and β-catenin. (G) Co-immunoprecipitation of the interaction between TCF4 and β-catenin. (H) Representative image and quantification of organoids generated from GBx+AOM/DSS mice treated with control, GUDCA, or GUDCA+OCA.

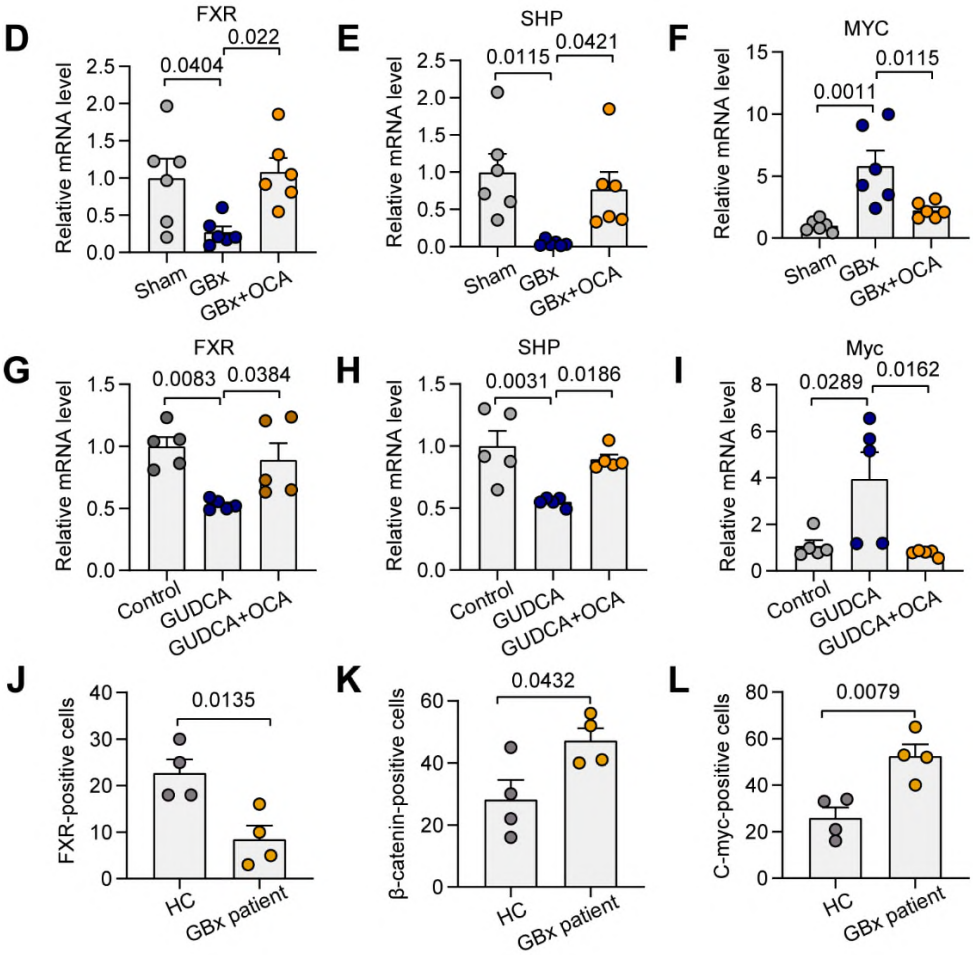


Fig. S20. FXR/β-catenin signaling is altered after OCA treatment in GBx mice or GUDCA-treated intestinal organoid, related to Fig.7. (D-F) Relative expression of FXR (D), SHP (E), and MYC (F) in each group. (G-I) Relative expression of FXR (G), SHP (H), and MYC (I) in intestinal organoid treated with GUDCA and OCA. (J-L) Quantitative analysis of FXR, β-catenin, and c-Myc IHC staining in healthy controls and patients with cholecystectomy.

MINOR COMMENTS & IMPROVEMENTS:

1. A detailed statistical analysis of CRC risk factors should be compared versus GBx contribution. If this study has previously done it should be referenced and well discussed. This should include the mutational status of the Wnt pathway on its main components: APC, CTNNB1, AXIN1/2, FBXW7, RNF43 and ZNRF3.

Response: We highly appreciate the valuable suggestion provided. A number of risk factors for CRC have been identified, including a history of CRC in a first-degree relative, an unhealthy diet, high alcohol consumption, physical inactivity, and obesity

(Gut. 2023; 72(2): 338-344; Clin Gastroenterol Hepatol. 2022; 20(6): 1229-1240.e5.). In our manuscript, we have included the demographic data of both cholecystectomy patients and healthy controls in Supplementary Table 1. Our findings revealed no significant changes in these indicators related to CRC risk factors between the two groups. Notably, previous clinical investigations demonstrated cholecystectomy is associated with an elevated risk of colorectal cancer ***(Gastroenterology 1993;105:142-7; PLoS One 2017;12: e0181852).*** In the current study, we observed that cholecystectomy is capable of promoting colorectal tumorigenesis in a manner dependent on the gut microbiota.

In CRC, genetic and epigenetic alterations influenced by lifestyle, environmental, and dietary factors drive carcinogenesis by activating oncogenes and inactivating tumor suppressor genes ***(Nat Rev Gastroenterol Hepatol. 2016; 13(12): 691-706).*** And most colorectal cancers harbour mutations in the tumor suppressor gene adenomatous polyposis coli (APC) and resultant hyperactivation of WNT signaling. ***(Nat Rev Microbiol. 2012; 10(8): 575-82).*** In our manuscript, we found that cholecystectomy promotes colorectal tumorigenesis in both AOM-DSS and APC $^{min/+}$ mice model (Fig.2 and Fig. S2). Notably, KEGG analysis indicated that the Wnt signaling pathway was involved in our model (Fig. 6E). Moreover, GBx treatment induced higher expression of Myc, a target gene of the Wnt/β-catenin pathway (Fig. 6F). These results demonstrated that cholecystectomy promotes colorectal carcinogenesis by activating the Wnt/β-catenin signaling pathway. We have diligently included the aforementioned data in the revised manuscript, specifically on Page 8 and 15.

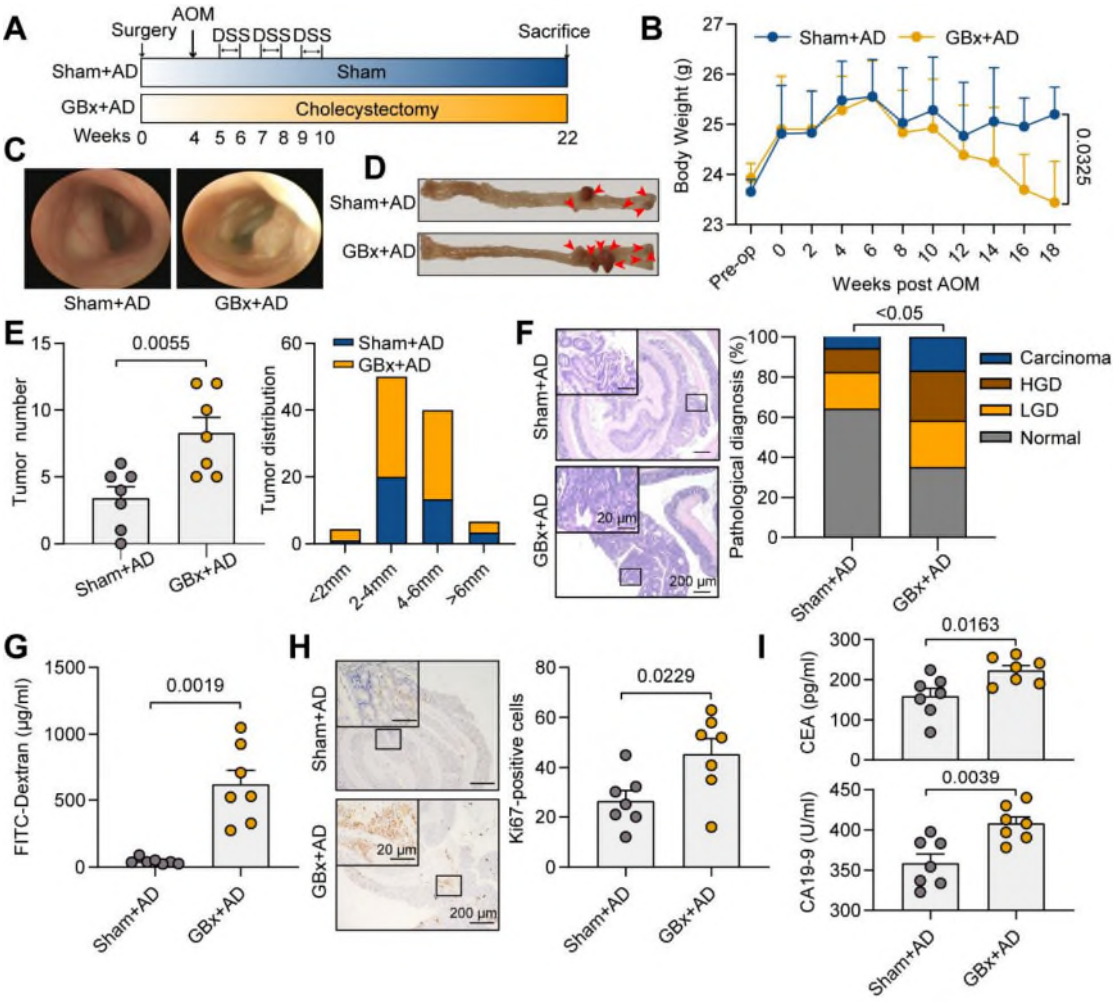


Fig. 2. Cholecystectomy promotes colorectal tumorigenesis in C57BL/6 mice. (A) Schematic diagram showing the experimental design and timeline. (B) The body weight of mice. (C) Representative colonoscopy images of mice. (D) Colonic morphologies. Each red arrow points at one tumor location. (E) Tumor number (left) and tumor size distribution (right). (F) Representative H&E staining of mouse colons. (G) Intestinal permeability measured by FITC-dextran. (H) Representative images and semiquantitative analysis of Ki67 staining of mouse colons. (I) Serum CEA (upper) and CA-199 (lower) levels in the two groups.

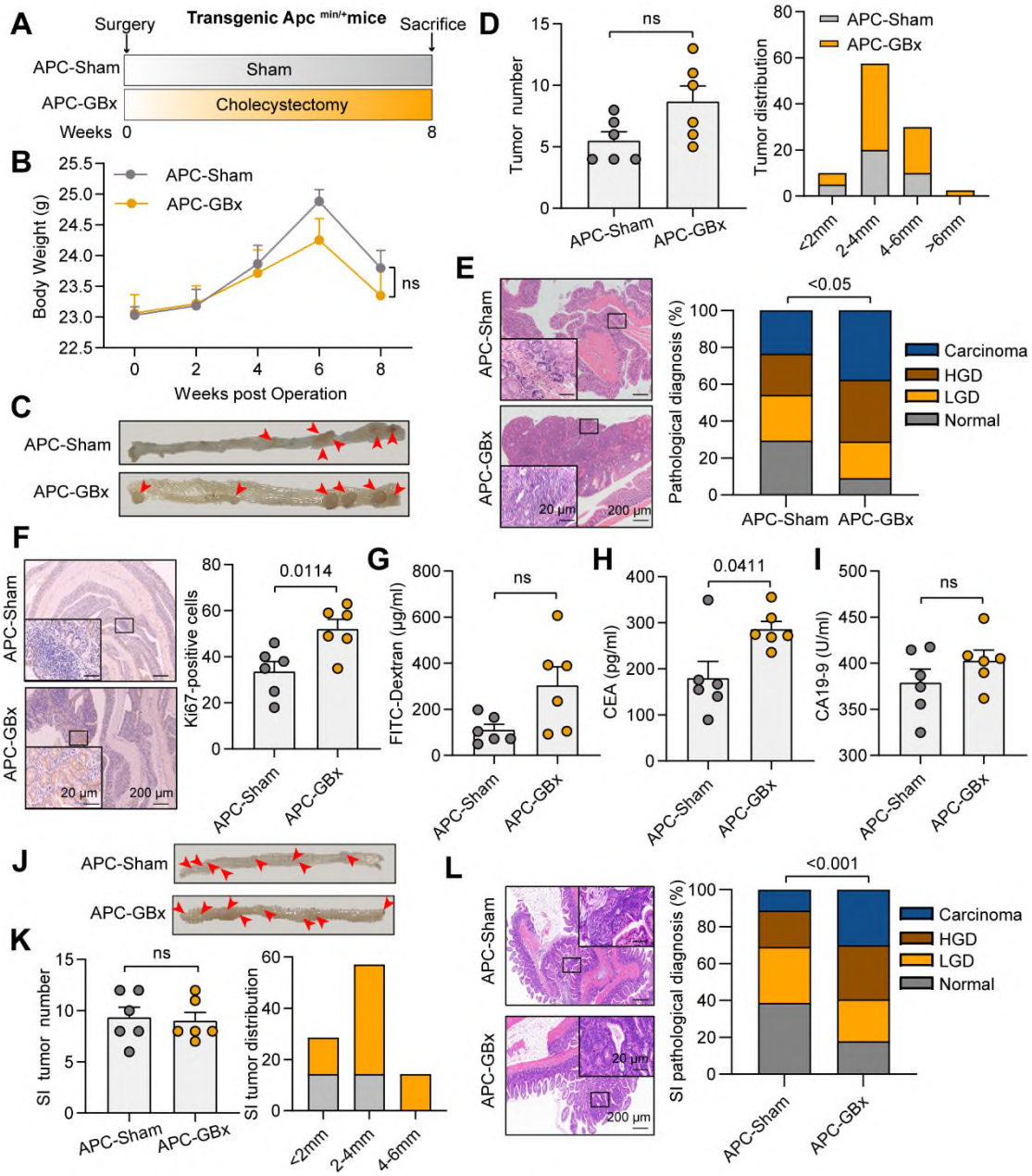


Fig. S2. Cholecystectomy facilitates colorectal tumorigenesis in APC$^{min/+}$ mice. (A) Schematic overview of the treatments in the APC$^{min/+}$ mouse model following cholecystectomy. (B) The body weight of mice throughout the experimental period. (C) Representative images of the colon in APC$^{min/+}$ mouse. (D) Average tumor number (left) and tumor size distribution (right) in APC$^{min/+}$ mice within the sham or cholecystectomy group. (E) Representative images of H&E staining (left) and semiquantitative analysis of the pathological score (right) in APC$^{min/+}$ mice. (F) Representative images of Ki67 staining of mouse colons and semiquantitative analysis. (G) Intestinal permeability measured by FITC-dextran in the above mice. (H and I) Serum levels of CEA and CA-199 (I) in mice. (J) Representative images of the intestine of APC$^{min/+}$ mouse at sacrifice. (K) Average tumor number (left) and tumor size distribution (right) in intestine of APC$^{min/+}$ mice. (L) Representative images of HE staining (left) and semiquantitative analysis of the pathological score (right) in the intestine of APC$^{min/+}$ mice.

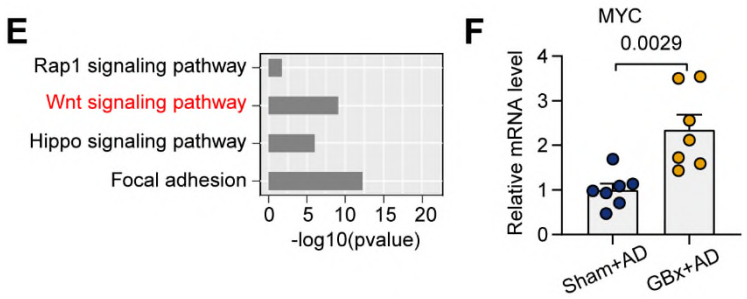


Fig. 6. FXR/β-catenin signaling is involved in cholecystectomy-related colorectal tumorigenesis. (E) KEGG pathway analysis of Sham+AOM/DSS and GBx+AOM/DSS mice. P values were adjusted by BH method to control FDR. FDR-adjusted P < 0.05 was shown. (F) Relative expression of Myc in Sham+AOM/DSS and GBx+AOM/DSS mice.

2. Testing the capacity of a R. gnavus strain with loss-of-function mutation or deletion of 7beta-HDSH on tumorigenesis will be relevant to prove its role in producing oncogenic metabolite GUDCA.

Response: In our quest to elucidate the role of bacterial bile salt hydrolase (BSH) and 7β-hydroxysteroid dehydrogenase (HSDH) activity in GBx-mediated colorectal cancer (CRC), we conducted a series of experiments employing BSH inhibitor riboflavin and 7β-HSDH inhibitor UDCA, respectively. Notably, administration of *B. breve* resulted in a reduction of GBx-induced colorectal tumors and tumor pathology. These effects were subsequently reversed upon treatment with the BSH inhibitor riboflavin (Fig.5H-I and Fig. S16A-B). Conversely, administration of *R. gnavus* led to an increase in tumor numbers, as well as proportions of adenocarcinoma, high-grade dysplasia, and low-grade dysplasia. However, these detrimental effects were ameliorated upon treatment with 7β-HSDH inhibitors UDCA (Fig.5H-I and Fig. S16A-B).

These recent findings have been appropriately incorporated into the revised manuscript, specifically on Page 14.

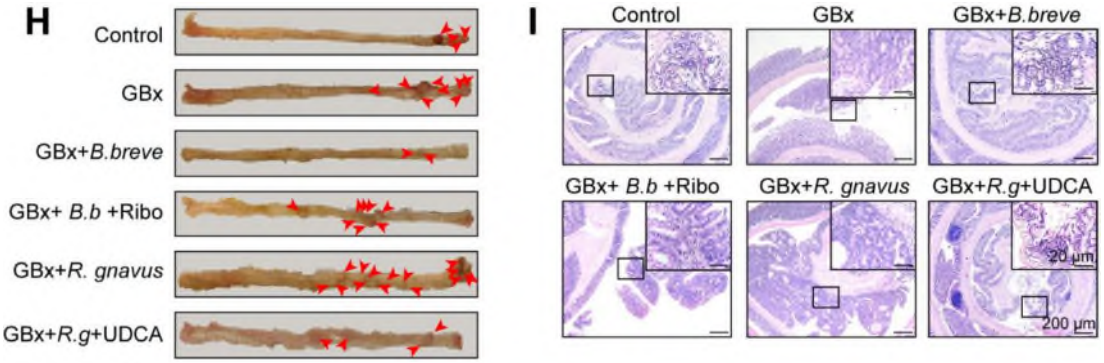


Fig. 5. *B. breve*- and *R. gnavus*-mediated bile acid metabolism is involved in colorectal tumorigenesis. (H-I) Representative images of mouse colon (H) and H&E staining (I) after *B. breve* or *R. gnavus* gavage, treated with BSH inhibitors riboflavin or 7β-HSDH inhibitors UDCA, respectively.

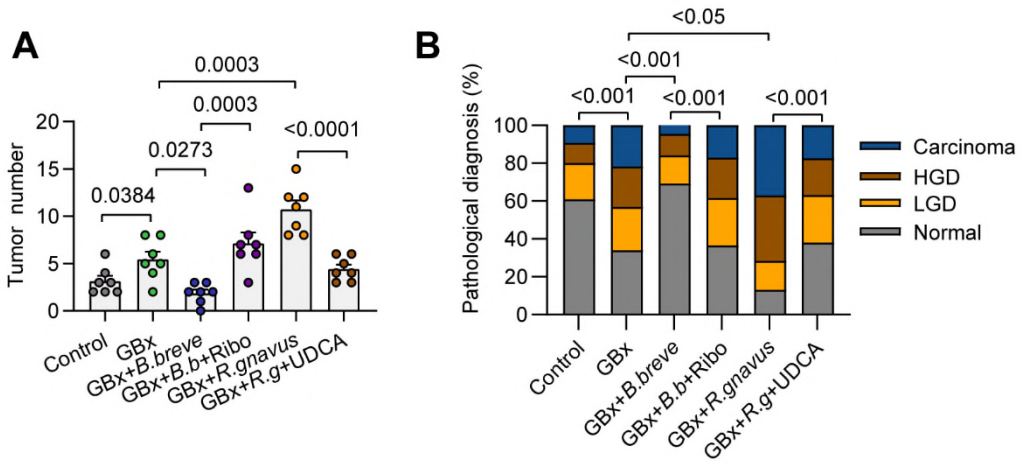


Fig. S16. Tumor number and quantitative analysis of H&E staining in mice, related to Fig. 5. (A) Average tumor number in mice after *B. breve* or *R. gnavus* gavage, treated with BSH inhibitors riboflavin or 7β-HSDH inhibitors UDCA, respectively. (B) Quantitative analysis of H&E staining in each group.

3. Does GBx reduce the methylation of FXR gene and its consequent increased expression?

Response: We express our sincere appreciation for the valuable comment provided. TUDCA and GUDCA have been shown to antagonize the FXR (***J Clin Invest. 2020; 130(1): 438-450. Nat Med. 2018; 24(12): 1919-1929***). Here, we observed an increase in TUDCA levels accompanied by FXR signaling inhibition following cholecystectomy in mice (Fig. 6B-D). Additionally, treatment with GUDCA significantly downregulated FXR and SHP expression in intestinal epithelial organoids (Fig. S20G-H). Based on previous studies and our own data, we posit that TUDCA or GUDCA may promote GBx-mediated colorectal tumorigenesis through the inhibition of FXR signaling. (attached below)

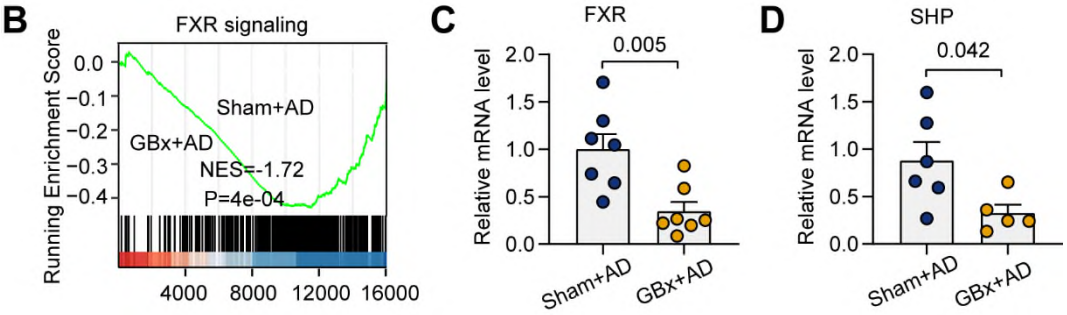


Fig. 6. FXR/β-catenin signaling is involved in cholecystectomy-related colorectal tumorigenesis. (B) GSEA of the changes in the FXR pathway genes between Sham+AOM/DSS and GBx+AOM/DSS group. (C-D) Relative expression of FXR (C) and SHP (D) in two group.

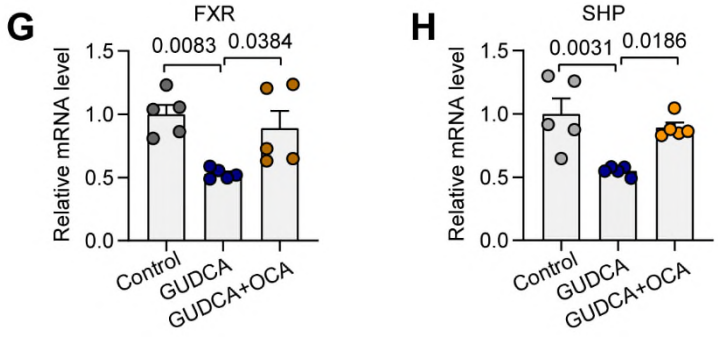


Fig. S20. FXR/β-catenin signaling is altered after OCA treatment in GBx mice or GUDCA-treated intestinal organoid, related to Fig. 7. (G-H) Relative expression of FXR (G) and SHP (H) in intestinal organoid treated with GUDCA and OCA.

The regulation of gene expression of FXR involves multiple mechanisms. ***(Biochim Biophys Acta. 2011; 1812(8): 842-50).*** And the ligand activation mechanism is the most crucial one ***(Mol Cell. 2003; 11(4): 1079-92; Mol Cell. 2003; 11(4): 1093-100).*** Synthetic or natural ligands and coactivator peptides bind to the LBD of FXR, resulting in a conformational change in FXR. This conformational alteration leads to the release of bound corepressor proteins and the recruitment of coactivator proteins to the DNA-binding site, thereby regulating the expression of FXR target genes. Whether FXR methylation or other epigenetic regulatory mechanisms are involved in colorectal tumorigenesis mediated by cholecystectomy is an important question that we will further explore in future work.

4. How GBx promotes the Wnt pathway? Any molecular result has been provided to explain such connection. This would improve the study significantly.

Response: We express our sincere appreciation for the insightful comment provided. In our manuscript, we found that Wnt/β-catenin signaling was upregulated after cholecystectomy, which could be reversed by FXR agonist OCA. The interaction between Wnt/β-catenin signaling and nuclear receptors has garnered considerable attention in cancer research ***(Acta Pharm Sin B 2024;14:1241-1256)***. Nuclear FXR has been reported to interact with β-catenin to form a complex, which subsequently interferes the transcriptional activity of the β-catenin/TCF. Inhibition of FXR disrupts the binding of catenin to TCF4 ***(Cell Death Dis 2020;11:640)***. Our results showed that the binding of FXR and β-catenin decreased, while the binding of β-catenin and TCF4 increased after cholecystectomy. Thus, our study reveals a non-transcriptional mechanism of FXR in cholecystectomy-mediated colorectal tumorigenesis, whereby FXR expression was downregulated after cholecystectomy, the interaction of FXR/β-catenin was subsequently disrupted, thus Wnt/β-catenin signaling was activated to promote colorectal tumorigenesis.

Reviewer #3 (Remarks to the Author):

In this manuscript, the authors describe changes in microbiota and fecal bile acid composition in post-cholecystectomy (GBx) patients. The authors link these changes to increased tumor formation in two different colorectal cancer (CRC) mouse models and altered FXR/beta-catenin signaling as a major mechanism for GBx-enhanced CRC. However, the mechanistic aspects of these changes, in particular, the physical interaction of beta-catenin and FXR (PMID 28714273) are not further explored in the manuscript, which we feel are essential to provide conceptual advance and novelty.

A few points to consider:

1) Patients undergoing cholecystectomy are usually suffering from bile duct obstruction (cholestasis) or other medical conditions. Is it possible that prior conditions also affect the incidence of CRC? This should at least be discussed.

Response: We greatly appreciate the valuable suggestion provided. Cholecystectomy was formerly perceived as being virtually harmless. Nevertheless, accumulating evidence suggests that there is a notable increase in the incidence of post-cholecystectomy syndromes and diseases related to metabolic syndrome ***(Clin Gastroenterol Hepatol 2023;21:940-948.e2).*** A number of risk factors for CRC have been identified, including a history of CRC in a first-degree relative, an unhealthy diet, high alcohol consumption, physical inactivity, and obesity ***(Gut. 2023; 72(2): 338-344; Clin Gastroenterol Hepatol. 2022; 20(6): 1229-1240.e5.).*** In our manuscript, we have included the demographic data of both cholecystectomy patients and healthy controls in Supplementary Table 1. Our findings revealed no significant changes in these indicators related to CRC risk factors between the two groups. Notably, previous clinical investigations demonstrated cholecystectomy is associated with an elevated risk of colorectal cancer ***(Gastroenterology 1993;105:142-7; PLoS One 2017;12: e0181852),*** while the underlying mechanism remains unidentified. In the current study, we observed that cholecystectomy is capable of promoting colorectal tumorigenesis in a manner dependent on the gut microbiota.

2) The authors performed Xeno-FMT to rescue CRC formation (human derived microbiome to mouse). There are major differences in bile acid metabolism and microbiota composition between human and mouse that should be considered.

Response: We greatly appreciate the insightful comment provided. In our study, we investigated the abundance of *B. breve* and *R. gnavus* in the group that underwent Fecal Microbiota Transplantation (FMT) using feces from cholecystectomy patients. Our data

analysis revealed a significant decrease in the abundance of *B. breve* (Fig. S6A), accompanied by a substantial increase in the abundance of *R. gnavus* (Fig. S6B) in this group. Additionally, when mice feces were transplanted, we observed a reduction in the abundance of *B. breve* in the FMT-GBx group (Fig. S7A), while the abundance of *R. gnavus* was found to be increased (Fig. S7B). These new findings have been diligently incorporated into the revised manuscript, specifically on Page 11.

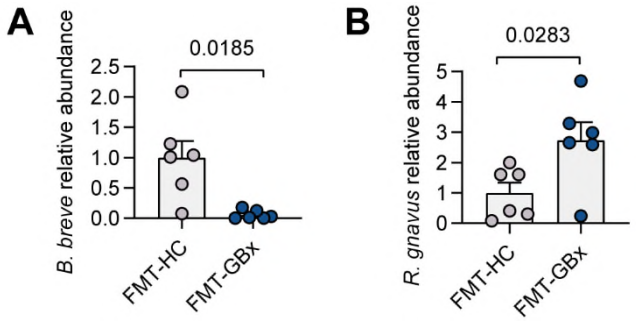


Fig. S6. FMT derived from GBx patients promotes colorectal tumorigenesis, related to Fig. 4. (A) qPCR analysis of *B. breve* abundance after FMT experiments. (B) qPCR analysis of *R. gnavus* abundance between FMT-HC and FMT-GBx group.

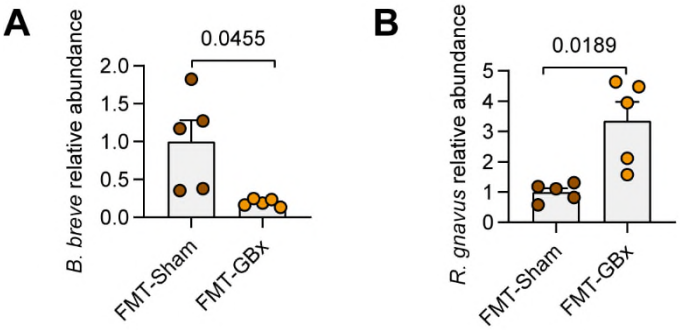


Fig. S7. FMT derived from tumor-bearing GBx mice promotes colorectal tumorigenesis, related to Fig. 4. (A-B) qPCR analysis of *B. breve* (A) and *R. gnavus* (B) abundance after mouse FMT experiments.

Furthermore, we previously observed that the GUDCA was elevated in the cholecystectomy population. It is important to note that due to the predominance of the taurine form of conjugated bile acid in mice, we were unable to detect significant levels of GUDCA in both mouse models. However, we did observe higher levels of TUDCA in both mouse models following cholecystectomy, as depicted in Fig. S11C and S12C. These results indicate that we have detected changes in key bacteria, namely *B. breve* and *R. gnavus*, and key bile acids, namely GUDCA and TUDCA, in both cholecystectomy populations and mice.

Please refer to the attached document for additional information regarding these findings.

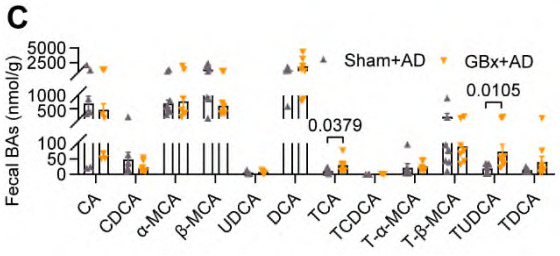


Fig. S11. Fecal bile acid profile in mice after cholecystectomy with AOM/DSS treatment. (C) Fecal bile acid profiles between the Sham+AOM/DSS and GBx+AOM/DSS mice.

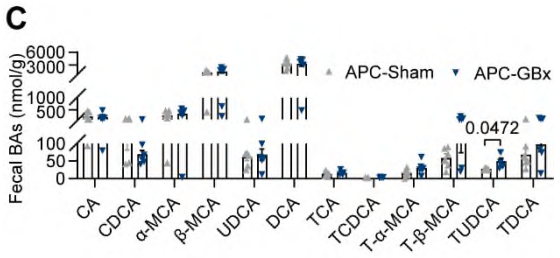


Fig. S12. Bile acid profile after cholecystectomy in APC$^{min/+}$ mice. (C) Fecal bile acid profiles in APC$^{min/+}$ mice with or without cholecystectomy.

3) Are reduced FXR expression/increased beta catenin expression accompanied by reduced FXR-beta catenin interaction only specific to tumor tissue or a common feature of GBx colon (non-tumor tissue)?

Response: We express our sincere appreciation for the insightful comment provided. In response to this feedback, we have made the necessary additions to our manuscript. Specifically, we have included a comprehensive description of the original Fig. 5 in both the Methods and Results sections (Page 15). It is important to note that the experiments were conducted using tumor-bearing mice. For the molecular biochemical experiment, such as RNA-seq and PCR analysis, we utilized tumor tissues obtained from the AOM/DSS mouse model.

Additionally, in accordance with the reviewer's suggestion, we investigated the expression of the target genes in naïve mice without AOM/DSS treatment. Notably, we observed a decrease in the expression of FXR (Fig. S17D) and its target gene SHP (Fig. S17E) decreased following GBx treatment. Conversely, the expression of Myc (Fig. S18F) was found to be increased. These findings provide valuable insights into the molecular changes associated with GBx in relation to these specific genes. Please refer to the attached document for a visual representation of these results.

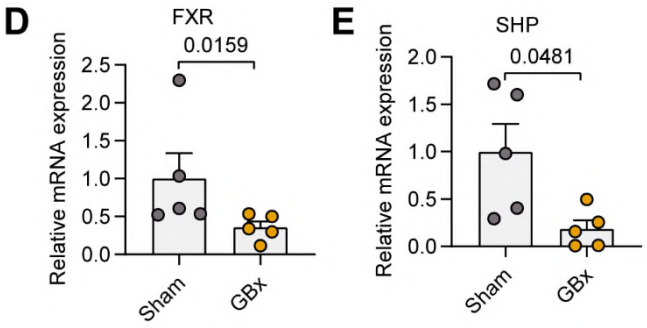


Fig. S17. The relative expression of bile acid receptors in Sham and GBx mice with or without AOM/DSS treatment, related to Fig. 6. (D) Relative expression of FXR in Sham and GBx mice. (E) Relative expression of SHP in each group.

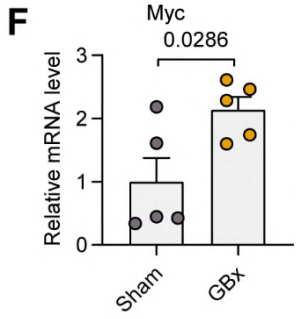


Fig. S18. The relative expression of target genes of Wnt/β-catenin signaling in GBx mice after AOM/DSS treatment, related to Fig. 6. (F) Relative expression of Myc in Sham and GBx mice.

4) Is GUDCA a major bile acid affecting CRC formation post-GBx at physiological concentrations?

Response: We sincerely appreciate the valuable and constructive comment provided. It is widely acknowledged that the majority of bile acids in the gut exist in a deconjugated form. In our study, we observed a significant increase in the ratio of conjugated to unconjugated bile acids (Fig. S10B), as well as a notable elevation in several conjugated bile acids in the GBx group (Fig. S10C), which is consistent with the previous report **(*Front Microbiol. 2022; 13: 800604*)** and highlight the impact of cholecystectomy on bile acid metabolism. Importantly, the complexity of bile acid metabolism is further exacerbated by different intervention factors following cholecystectomy, as confirmed by previous studies (***Gut microbes, 2023; 15(1): 2168101; Nutrients; 2023; 15(17): 3829***) and our own data.

Notably, previous investigations have indicated that cholecystectomy does not lead to alterations in the cholic acid (CA) or deoxycholic acid (DCA) pools in patients during short-term and long-term follow-up **(*J Clin Invest. 1989; 83(5):1541-50; Hepatology. 1995;21(1):41-5***). While some studies have reported an increase in serum DCA levels following cholecystectomy, a substantial body of research has consistently shown no changes in fecal DCA, LCA, and CDCA levels after cholecystectomy **(*Front Microbiol. 2022: 18: 13: 800604; Nutrients. 2023; 15 (17): 3829***), which is consistent with our

findings. Importantly, we observed an elevation in fecal GUDCA levels in patients who underwent cholecystectomy (Fig. 1H), and Z-score analysis further demonstrated the significance of this difference (Fig.1I). Although the presence of concomitant AOM/DSS intervention or APC genetic manipulation may complicate the bile acid profiles, we consistently observed changes in TUDCA levels across different models following cholecystectomy (Fig. S11C and Fig. S12C), as depicted in the attached document.

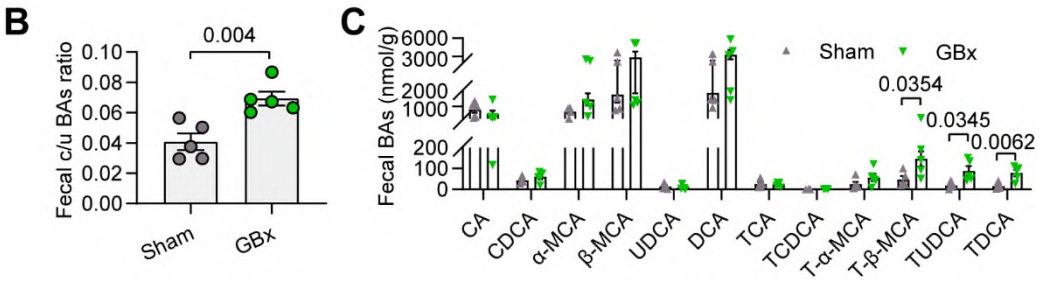


Fig. S10. Bile acid profile in mice after cholecystectomy. (B) The ratio of conjugated to unconjugated bile acids in fecal samples between the two groups. (C) Fecal bile acid profiles in the two groups.

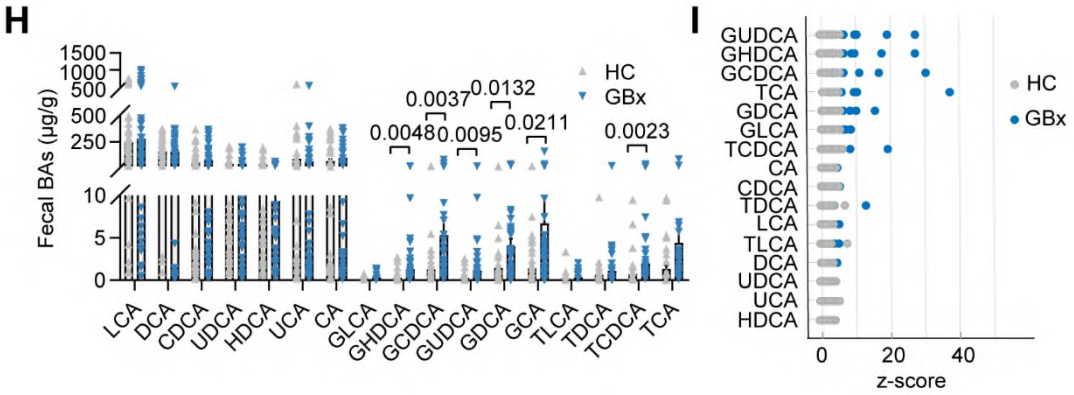


Fig. 1. The gut microbiota and bile acid profiles in cholecystectomy patients. (H) Fecal bile acid profiles in health controls and GBx patients. (I) Z-score of the bile acids.

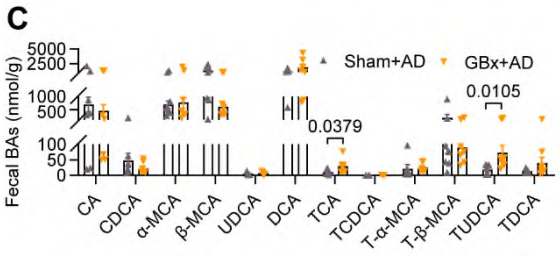


Fig. S11. Fecal bile acid profile in mice after cholecystectomy with AOM/DSS treatment. (C) Fecal bile acid profiles between the Sham+AOM/DSS and GBx+AOM/DSS mice.

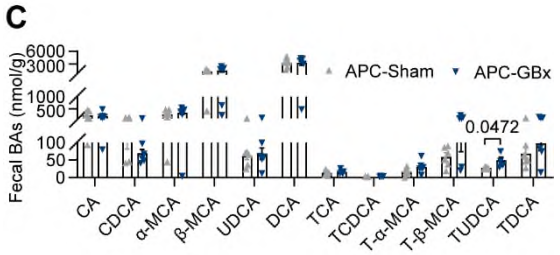


Fig. S12. Bile acid profile after cholecystectomy in APC$^{min/+}$ mice. (C) Fecal bile acid profiles in APC$^{min/+}$ mice with or without cholecystectomy.

Dear editor and reviewers,

We extend our utmost gratitude for the invaluable feedback and suggestions provided. These comments have proven to be immensely valuable in enhancing the quality of our manuscript. We have diligently incorporated the necessary revisions and supplemented the document with the requested additional data. Please find below a comprehensive point-by-point response to each comment:

Reviewer #1 (Remarks to the Author):

The authors have provided answers, which raise the following comments:

1. The causal relationship between cholecystectomy and colorectal cancer (CRC) or other complications in humans is still debated, whereas it is presented as well established.

In their answer, the authors provide two references, a review on post-cholecystectomy syndrome and meta-analysis on colorectal cancer (CRC), none of which have been included in the introduction of the revised manuscript.

Response: We express our sincere gratitude for the constructive comments provided by the esteemed reviewers. We have added the relevant references (Reference 2 and 6) in the introduction section in the revised manuscript.

2. The information regarding the patients provided in Table S1, is very limited so that there could be many confounding factors notably metabolic factors, which could explain the differences in gut microbiota and bile acid profile between the two groups, besides cholecystectomy. The time elapsed since cholecystectomy apparently ranges between 1 and 20 years, which makes the study group very heterogeneous, considering the adaptation of bile acids metabolism which may occur in patients post-cholecystectomy. Two patients in the group of cholecystectomy developed CRC, whereas it is not specified if CRC occurred before or after cholecystectomy, and how long after if this is the case. In addition, the statistic method used to analyze patients'date should appear in the legend of Table S1.

No answer was provided regarding the occurrence of CRC before or after

cholecystectomy. Moreover, Table S1 shows that the only significant difference between the healthy and cholecystectomy groups according to chi-square test, was more frequent diarrhea in the cholecystectomy group whereas CRC occurrence was not significantly different between the two groups. Importantly, the post-operative time is of 9.08 ± 0.83 without unit, years presumably. It is very surprising that serum bile acids are increased 9 years after cholecystectomy (Fig. 1f) unless the patients have additional liver disease. Therefore, it would be essential to mention the indication of cholecystectomy and the liver status.

2.1 No answer was provided regarding the occurrence of CRC before or after cholecystectomy.

Response: We greatly appreciate the valuable and constructive comment. Actually, two patients in the group of cholecystectomy developed CRC after cholecystectomy. Specifically, one case occurred four years after cholecystectomy, and the other occurred six years after cholecystectomy. We have added the information in the revised manuscript on page 8.

2.2 Moreover, Table S1 shows that the only significant difference between the healthy and cholecystectomy groups according to chi-square test, was more frequent diarrhea in the cholecystectomy group whereas CRC occurrence was not significantly different between the two groups.

Response: Although the CRC occurrence was not significantly different between the two groups, we did find 2 out of 52 patients after cholecystectomy developed CRC, implying an upward trend of CRC incidence after cholecystectomy. However, due to the relatively small sample size, there was no significant difference between the two groups. Importantly, previous studies showed that the occurrence rate of CRC increased markedly after cholecystectomy ***(Br J Cancer. 2003; 88: 79-83; PLoS One. 2017; 12: e0181852; Front Med. 2022; 9: 1000563).*** Therefore, integrating the previous literature and our findings suggests that the risk of CRC occurrence increases after cholecystectomy.

2.3 Importantly, the post-operative time is of 9.08 ± 0.83 without unit, years presumably.

Response: The post-operative time is of 9.08 ± 0.83 years, and we have added the units in the revised Supplementary Table 1.

2.4 It is very surprising that serum bile acids are increased 9 years after cholecystectomy (Fig. 1f) unless the patients have additional liver disease. Therefore, it would be essential to mention the indication of cholecystectomy and the liver status.

Response: As the reviewer mentioned, the adaptation of bile acids metabolism may occur in patients post-cholecystectomy. Numerous studies have reported a significant increase in serum bile acid levels in patients who have undergone cholecystectomy ***(PloS One. 2015; 10: e0118478; Eur J Intern Med. 2018; 53: 3-11),*** which is consistent with our results. After cholecystectomy, the increase in serum bile acid levels can be attributed to several factors. Firstly, removing the gallbladder disrupts the normal circulation and metabolism of bile acids, which changes their distribution in the body, directly causing a rise in serum bile acid levels ***(Gut. 1978; 19: 640-9; Gut. 1973; 14: 753-62)***. Secondly, cholecystectomy interrupts the enterohepatic circulation of bile acids, leading to changes in the expression of genes related to bile acid synthesis. Concurrently, enhanced intestinal absorption of bile acids may occur, resulting in elevated serum bile acid levels ***(Compr Physiol. 2016; 6: 1549-77; Eur J Intern Med. 2018; 53: 3-11)***. Finally, after cholecystectomy, the continuous flow of bile into the intestine causes intestinal flora dysbiosis, which in turn affects the metabolism and reabsorption of bile acids, leading to an increase in serum bile acid levels ***(Front Microbiol. 2022; 13: 800604; Int J Surg. 2023; 109: 2585-2597; Gut Microbes. 2023; 15: 2168101***). We have added the above explanations and relevant references in the Discussion section.

In addition, we have added the information of liver function indicators and liver diseases that have occurred during the follow-up process into Supplementary Table 1. There were no significant differences in both liver function indicators and liver diseases between the two groups.

Supplementary Table 1. Demographic and clinical characteristics of health control

and cholecystectomy patients.

Parameter	Health (N=45)	Cholecystectomy (N=52)	P Value
Gender, female, No, (%)	28 (62.22%)	32 (61.54%)	0.95
Age (years)	48.53±1.31	51.15±1.42	0.68
Postoperative time (years)		9.08±0.83	
Height (m)	1.62±1.28	1.61±1.18	0.85
Weight (Kg)	55.24±1.34	55.13±1.25	0.76
BMI (Kg/m2)	24.91±0.41	25.30±0.26	0.63
Clinical symptoms			
Acid Reflux, No. (%)	4 (8.89%)	5 (9.62%)	0.90
Diarrhea, No. (%)	2 (4.44%)	10 (19.23%)	0.03
Abdominal Distension, No. (%)	5 (11.11%)	7 (13.46%)	0.73
Abdominal Pain, No. (%)	2 (4.44%)	8 (15.38%)	0.08
Constipation, No. (%)	3 (6.67%)	4 (7.69%)	0.85
Liver function test			
ALT (U/L)	22.39±1.55	28.40±3.09	0.53
AST (U/L)	22.16±1.05	24.37±1.22	0.66
ALP (U/L)	87.90±3.52	90.89±4.89	0.41
GGT(U/L)	24.66±1.99	30.93±2.45	0.29
TBIL(μmol/L)	10.03±0.75	10.66±0.60	0.24
Comorbidities			
HBP, No. (%)	8 (17.78%)	10 (19.23%)	0.85
HLP, No. (%)	5 (11.11%)	7 (13.46%)	0.73
DM, No. (%)	3 (6.67%)	6 (11.54%)	0.41
NAFLD, No. (%)	3 (6.67%)	4 (7.69%)	0.85
AILD, No. (%)	1 (2.22%)	2 (3.85%)	0.65
Cirrhosis, No. (%)	0 (0.00%)	1 (1.92%)	0.35
IBD, No. (%)	1 (2.22%)	2(3.85%)	0.65
CRC, No. (%)	0 (0.00%)	2 (3.85%)	0.18

Data were present as the Mean ± SE, P value was determined by chi-square test. ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, gamma-glutamyl

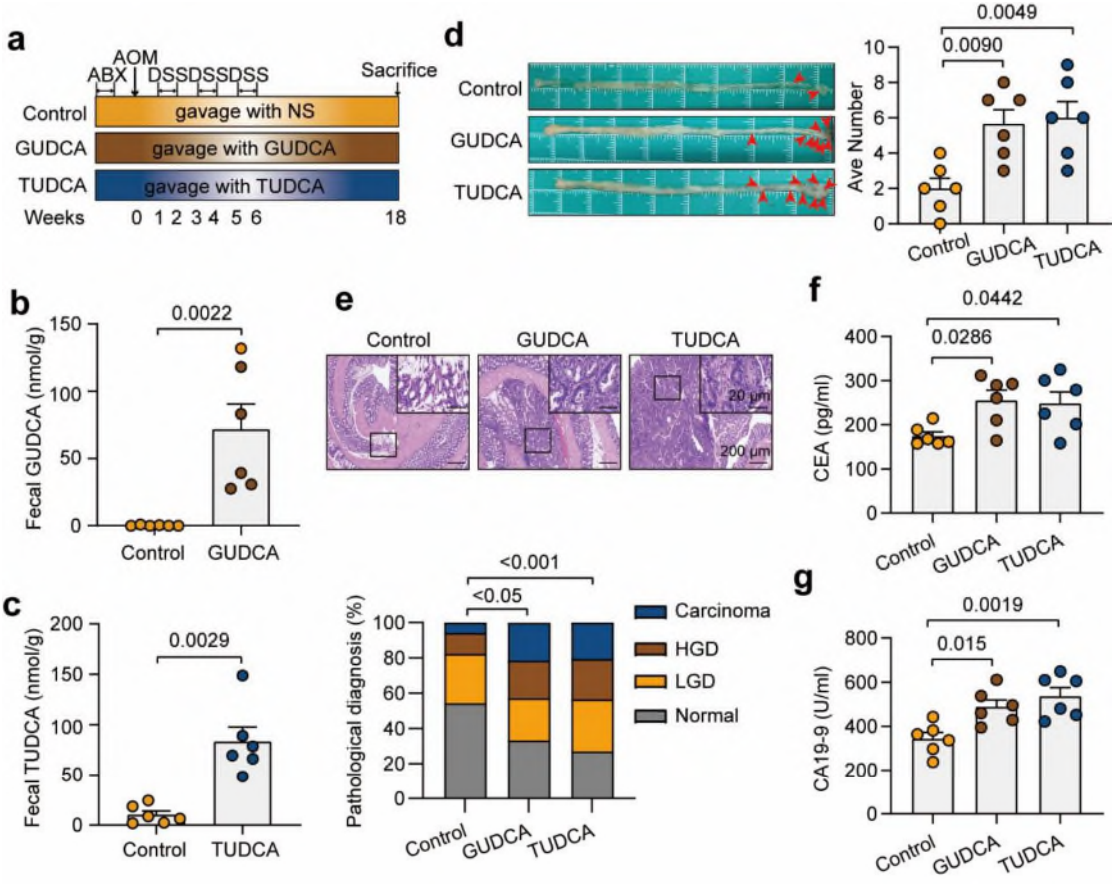
transferase; TBIL, total bilirubin; HBP, high blood pressure; HLP, hyperlipidemia; DM, diabetes; NAFLD, non-alcoholic fatty liver disease; AILD, autoimmune liver disease; IBD, inflammatory bowel disease; CRC, colorectal cancer. Source data are provided as a Source Data file.

3. The mechanism whereby cholecystectomy promotes CRC in mice was investigated by performing GUDCA gavage, whereas it is TUDCA which increases after cholecystectomy in mice. Likewise, whereas B. breve is decreased following cholecystectomy in mice, it seems to be increased in humans (Fig. 1C). Generally, figure legends are not self-explanatory, which makes the interpretation of data difficult. The authors performed additional experiments showing that TUDCA has the same effect as GUDCA. Yet, if the objective is to show that the effect of GBx is mediated by TUDCA, TUDCA should reproduce the effect of GBx instead of being combined with GBx. It is unclear why the increase in R.gnavus in cholecystectomized patients is not shown in Fig. 1, but shown later in Fig. S4g. The presentation of gut microbiota data remains confusing in the text between GBx alone, GBx + AOM/DSS, APC-GBx, and should not be shown as supplementary, but as a full figure of both mice and patients data as in the response to reviewer (Fig. S4F-L). And still, B.breve is increased in humans (fig. 1C) Figure legends are still not self-explanatory. For example, what lesions does Fig. 3C precisely show.

3.1 The authors performed additional experiments showing that TUDCA has the same effect as GUDCA. Yet, if the objective is to show that the effect of GBx is mediated by TUDCA, TUDCA should reproduce the effect of GBx instead of being combined with GBx.

Response: We highly appreciate the valuable comment addressed. We have conducted these experiments before, and added the relevant data of TUDCA or GUDCA in the absence of GBx in colorectal tumorigenesis as follows. Mice that received TUDCA or GUDCA treatment developed an increased number of tumors (Supplementary Fig. 16d), a higher histopathological grading (Supplementary Fig. 16e), and increased levels of CEA and CA19-9 (Supplementary Fig. 16f-g). These results indicated that TUDCA or

GUDCA in the absence of GBx could reproduce the effect of GBx in colorectal tumorigenesis. We have added these new data in Supplementary Fig. 16 in the revised manuscript, specifically on Page 12.



Supplementary Fig 16. GUDCA and TUDCA gavage promotes colorectal tumorigenesis, related to Fig. 5. a Experimental design for mice orally gavaged with NS (control), GUDCA or TUDCA. **b** Fecal GUDCA level after GUDCA gavage (Mann-Whiteney *U* test). **c** Fecal TUDCA level after TUDCA gavage (Welch's *t* test). **d** Representative colonic morphologies and average tumor number in each group. **e** Representative images of H&E staining (up) and semiquantitative analysis (down). Histological slides showing normal, dysplastic mucosae and carcinoma in the experiment. The bottom scale bar is 200 μm and the top scale bar is 20 μm. **f-g** Serum CEA **(f)** and CA19-9 levels **(g)** in each group. n = 6/group. P values were determined by ANOVA for **d-g** and were indicated in each figure. NS: normal saline. Source data are provided as a Source Data file.

3.2 It is unclear why the increase in R.gnavus in cholecystectomized patients is not shown in Fig. 1, but shown later in Fig. S4g. The presentation of gut microbiota data

remains confusing in the text between GBx alone, GBx + AOM/DSS, APC-GBx, and should not be shown as supplementary, but as a full figure of both mice and patients data as in the response to reviewer (Fig. S4F-L). And still, B.breve is increased in humans (fig. 1C).

Response: In our study, we first analyzed the differences in gut microbiota between patients who had undergone cholecystectomy and healthy individuals (Fig. 4a-b). Through VIP analysis, we identified that *B. breve* was the bacteria with the most significant difference between the two groups (Fig. 4c). Subsequently, we analyzed the differences in gut microbiota in the mouse model (Fig. 4d-e), and VIP analysis revealed that *R. gnavus* was the bacteria with the most significant difference (Fig. 4f). We further verified the abundance of these two bacteria in both the human cohort and the GBx+AOM/DSS model. We observed that the abundance of *B. breve* significantly decreased, while *R. gnavus* significantly increased (Fig. 4g-j). To further verify that the abundance change is attributed to cholecystectomy, we tested the levels of these two bacteria in the GBx model and the APC-GBx mice model. Consistently, the abundance of *B. breve* significantly decreased, and the abundance of *R. gnavus* significantly increased (Fig. 4k-n) in these two mice models. Importantly, the growth of *B. breve* was inhibited in bile salts in a concentration-dependent manner (Supplementary Fig. 10a), while *R. gnavus* was resistant to bile salts (Supplementary Fig. 10b). Taken together, our data indicate that cholecystectomy can reduce the level of *B. breve* and increase the level of *R. gnavus*. According to the reviewer's suggestion, we consolidated these microbiota data into a full figure in Fig. 4 (attached below). Also, we added the above statements in the revised manuscript on Page 8-9.

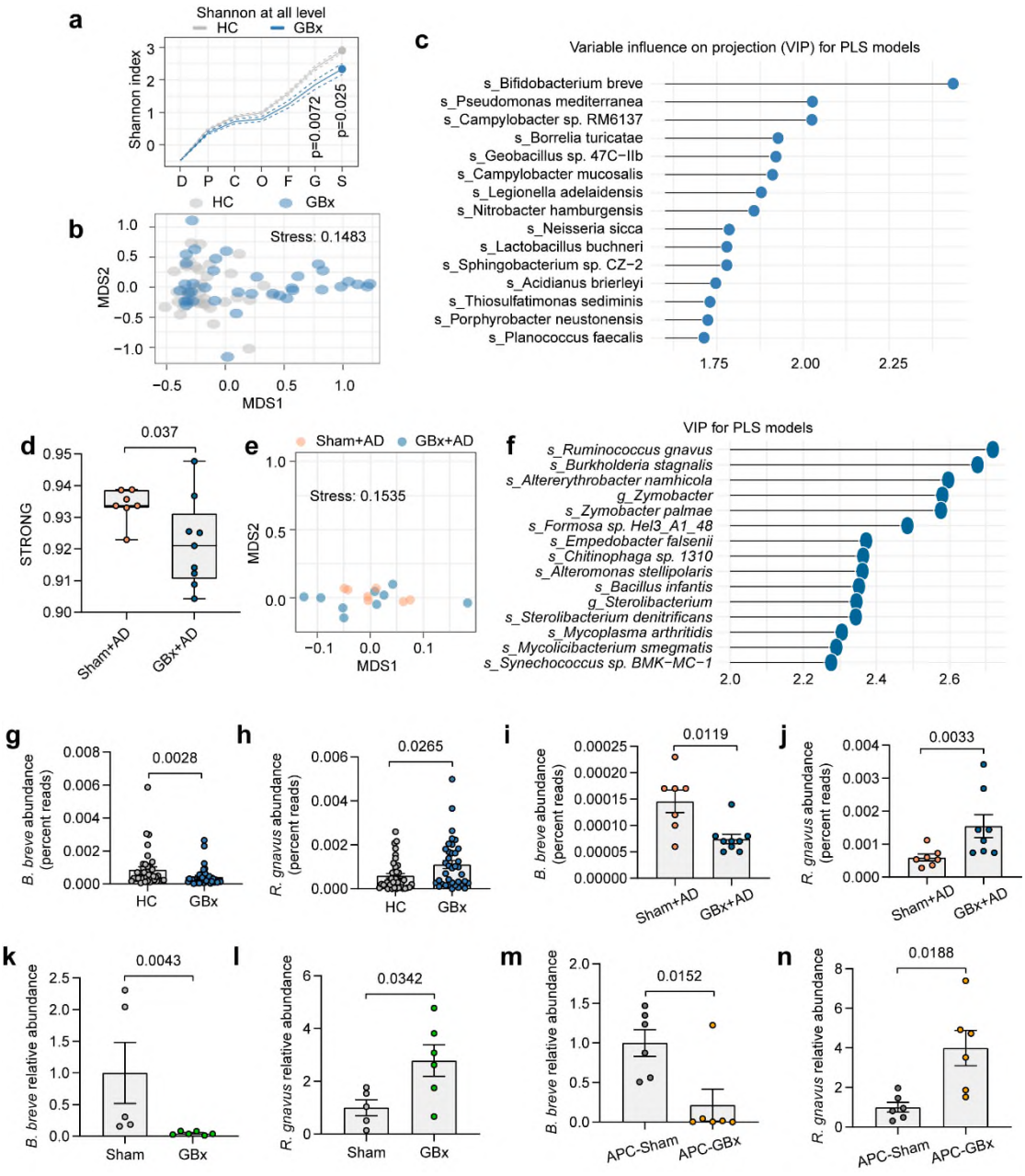


Fig.4. The gut microbiota profiles in cholecystectomy patients and mice models. a α-diversity of the gut microbiota at all levels in HC and GBx patients (Welch's *t* test). **b** NMDS of bacterial communities based on Bray-Curtis similarity. **c** VIP score of the PLS-DA in gut microbiota. **d** Index (α-diversity) of the gut microbiota between Sham+AOM/DSS and GBx+AOM/DSS mice. (Welch's *t* test, n = 7 vs 9). **e** NMDS of bacterial communities based on Bray-Curtis similarity (n =7 vs 9). **f** VIP score of the PLS-DA. VIP scores were used to rank the ability of different taxa to discriminate between the two groups. A taxon with VIP score > 2.0 was considered important in the discrimination (n = 7 vs 9). **g** Relative abundance (percent reads) of *B. breve* based on metagenomics sequencing. **h** The relative abundance of *R. gnavus* between healthy and cholecystectomy patients. **i** The relative abundance of *B. breve* between the

Sham+AD and GBx+AD group (n = 7 vs 9). **j** Relative abundance of *R. gnavus* between Sham+AOM/DSS and GBx+AOM/DSS mice (n = 7 vs 8). **k** qPCR analysis of *B. breve* abundance in Sham and GBx mice (n = 5 vs 6). **l** qPCR analysis of *R.gnavus* in Sham and GBx mice (*t* test, n = 5 vs 6). **m** qPCR analysis of *B. breve* abundance between APC-Sham and APC-GBx mice (n = 6/group). **n** qPCR analysis of *R. gnavus* in APC $^{min/+}$mice (Welch's t test, n = 6/group). *P* values were determined by Mann Whitney *U* test for **g-k, m**, and were indicated in each figure. NMDS, nonmetric multidimensional scaling; VIP, variable importance in projection. Source data are provided as a Source Data file.

3.3 Figure legends are still not self-explanatory. For example, what lesions does Fig. 3C precisely show.

Response: In addition, we revised the description of the figure legends in original Fig. 3c, which is now presented as Fig. 2c to make it self-explanatory. Here, the histological slides showed acute inflammation at 4 weeks and chronic inflammation at 10 weeks following AOM injection.

4. The English syntax needs to be thoroughly revised in particular to express quantitative data properly, e.g. "more severe tumor distribution" (lines 355-356) does not mean anything. The meaning of several other sentences is unclear, for example, lines 133 and 147: "tumor distribution…larger and wider"; line 147 "colorectal tumor distribution…greater ; line 200 more obvious CRC phenotype; line 393 "a major role in this process": which process does this refer to?; lines 396-397, "the transformation from CDCA to UDCA …has proven feasible"; lines 432-433 "Our results showed that the combination….to the control group". Line 432, reference 33 is not appropriate.

Language editing by Springer Nature improves reading of the manuscript.

Response: We appreciate the reviewers' recognition of this comment.

5. The number of replicates is not indicated for all quantitative data in figure legends. Details are also lacking regarding the histopathological analysis of the colon in mice. The score was the average of scores determined by two experts. Information is lacking

regarding the intra- and inter-observer variability in this scoring analysis? Information is also lacking regarding the distribution of lesions along the colon, i.e., proximal vs. distal. How were the different grades of histological severity found in each mouse taken into account. In addition, it is not specified which part of the colon or if the entire colon were used for RT-PCR analyses.

All the details provided in the response should be included in the manuscript. The number of replicates should exceed 3. If one goes in one direction, and the other two in the opposite direction, representative of three is almost random. The legend of representative images is misleading, e.g. Fig. 2c, 2f: it would be more appropriate to show carcinoma, HDG and LDG, which are all found in both Sham and GBx, the difference is only quantitative.

Response: We have incorporated all the details provided in the response into both the main text and supplementary materials of the revised manuscript. The number of replicates is indicated for all quantitative data in all figure legends and each experiment has been replicated over three times. We have added this in the Methods and figure legends. Furthermore, we also made the revisions to the descriptions of the figure legends for the original Fig.2c and Fig. 2f, which were now presented as Fig. 1c and Fig. 1f respectively, to enhance the comprehensibility of the figures.

6. Some statements like “after cholecysctectomy, the enterohepatic circulation of bile acid was accelerated…” (line 115) was not shown in the present study, and if this is the case, the proportion of conjugated bile acids should be decreased rather than increased as found here (line 118).

This statement has been corrected.

Response: We appreciate the reviewers' recognition of this comment.

7. All abbreviations should be defined.

There are still undefined abbreviations, e.g., BSH.

Response: We sincerely appreciate the comment raised. We have carefully checked the entire manuscript and defined all abbreviations in the revised manuscript.

In conclusion, the authors addressed several comments, but a focus on the most consistent data would be more convincing to propose a mechanism, whereby cholecystectomy may promote colorectal cancer .

Reviewer #3 (Remarks to the Author):

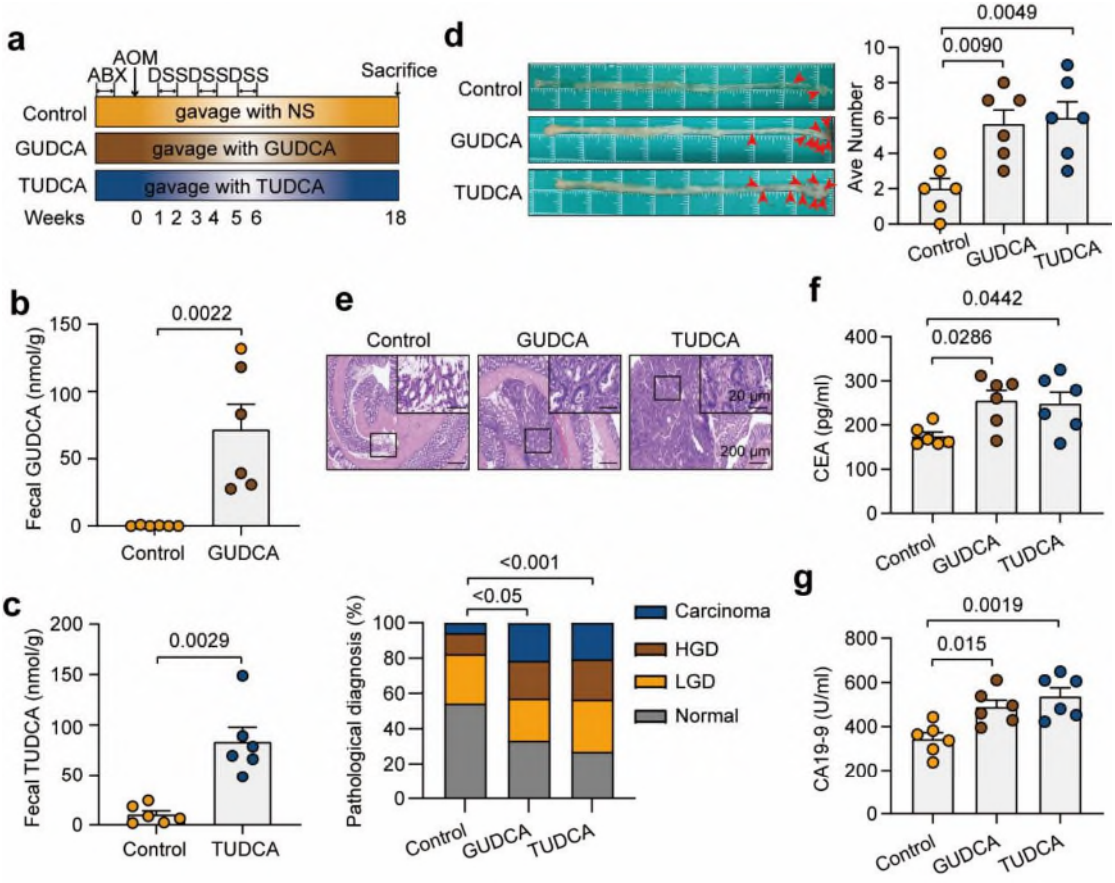
The authors have conducted a significant number of experiments that have substantially improved the overall quality of their manuscript. However, a few critical points remain to be addressed before the manuscript is suitable for publication.

1. GUDCA and TUDCA promotion of CRC was only done in the setting of GBx with supraphysiological addition of these bile acids. The question of whether or not physiological levels of these bile acids induced by GBx are themselves pro-tumorigenic remains unaddressed. Did the authors test if TUDCA or GUDCA addition in the absence of GBx can recapitulate GBx promotion of CRC? If so, this should be shown and discussed. Regardless, the authors should measure fecal levels of GUDCA and TUDCA after oral gavage for the experimental conditions they do show to understand how these relate to physiological levels induced by GBx.

Response: We highly appreciate the valuable suggestion provided. We have conducted these experiments before, and added the relevant data of TUDCA or GUDCA in the absence of GBx in colorectal tumorigenesis as follows. We observed that TUDCA or GUDCA treatment could develop an increased number of tumors (Supplementary Fig. 16d), a higher histopathological grading (Supplementary Fig. 16e), and increased levels of CEA and CA19-9 (Supplementary Fig. 16f-g). These results suggest that gavage of

either GUDCA or TUDCA in the absence of GBx can still promote the development of CRC. These new findings have been added to Supplementary Fig. 16 in the revised manuscript, specifically on Page 12.

Importantly, according to the reviewer's suggestion, we measured the levels of GUDCA or TUDCA in feces after gavage of GUDCA or TUDCA in the absence of GBx. The data showed that the fecal levels of GUDCA or TUDCA increased significantly (Supplementary Fig. 16b-c), which were comparable to the fecal levels of TUDCA after cholecystectomy (Supplementary Fig. 13c). Therefore, oral gavage of TUDCA or GUDCA for the experimental conditions is comparable to the physiological levels induced by cholecystectomy.



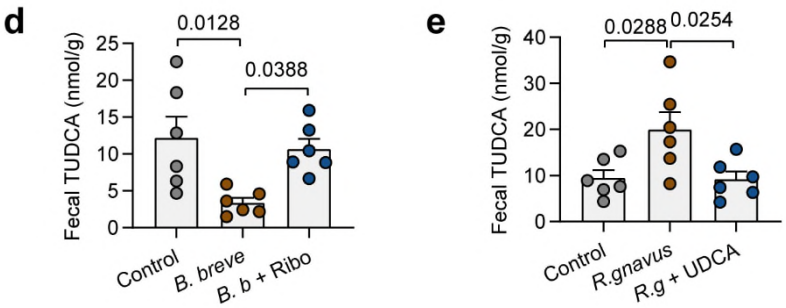
Supplementary Fig 16. GUDCA and TUDCA gavage promotes colorectal tumorigenesis, related to Fig. 5. a Experimental design for mice orally gavaged with NS (control), GUDCA or TUDCA. **b** Fecal GUDCA level after GUDCA gavage (Mann-Whiteney *U* test). **c** Fecal TUDCA level after TUDCA gavage (Welch's *t* test). **d** Representative colonic morphologies and average tumor number in each group. **e** Representative images of H&E staining (up) and semiquantitative analysis (down). Histological slides

showing normal, dysplastic mucosae and carcinoma in the experiment. The bottom scale bar is 200 μm and the top scale bar is 20 μm. **f-g** Serum CEA **(f)** and CA19-9 levels **(g)** in each group. *n* = 6/group. *P* values were determined by ANOVA for **d-g** and were indicated in each figure. NS: normal saline. Source data are provided as a Source Data file.

2. While the experiments using riboflavin to inhibit BSH in B. breve and UDCA to inhibit 7β-HSDH in R. gnavus are supportive of the concept that bacterial modulation of TUDCA levels underlie tumorigenic potential, these inhibitors can have pharmacologic impacts beyond modulation of bile acids. Ideally, the authors should measure fecal TUDCA levels in the presence of these inhibitors to support the validity of their mechanisms of action. If this cannot be done, additional confounding actions of riboflavin and UDCA should at least be discussed in the manuscript.

Response: We greatly appreciate the constructive comment. We have added the data to determine the fecal TUDCA levels in the presence of these two inhibitors. Gavage with *B. breve* reduces the fecal TUDCA level, while riboflavin can increase the TUDCA level mediated by *B. breve* (Supplementary Fig. S17d). Moreover, UDCA treatment can reverse the elevation of TUDCA levels mediated by *R. gnavus* (Supplementary Fig. S17e). These results indicate that bacterial modulation of TUDCA levels can indeed participate in cholecystectomy-mediated CRC oncogenesis.

We have added these new findings in Supplementary Fig 17 in the revised manuscript, specifically on Page 13.



Supplementary Fig. 17. The correlations of *B. breve* and *R. gnavus* with bile acids, and the fecal level of TUDCA with inhibitors administration, related to Fig. 5. d Fecal TUDCA level after *B. breve* gavage with or without riboflavin. **e** Fecal level of TUDCA following the gavage of *R. gnavus* in the

presence or absence of UDCA. *n* = 6/group. *P* values were determined by ANOVA for **d-e** and were indicated in each figure. Ribo, riboflavin. Source data are provided as a Source Data file.

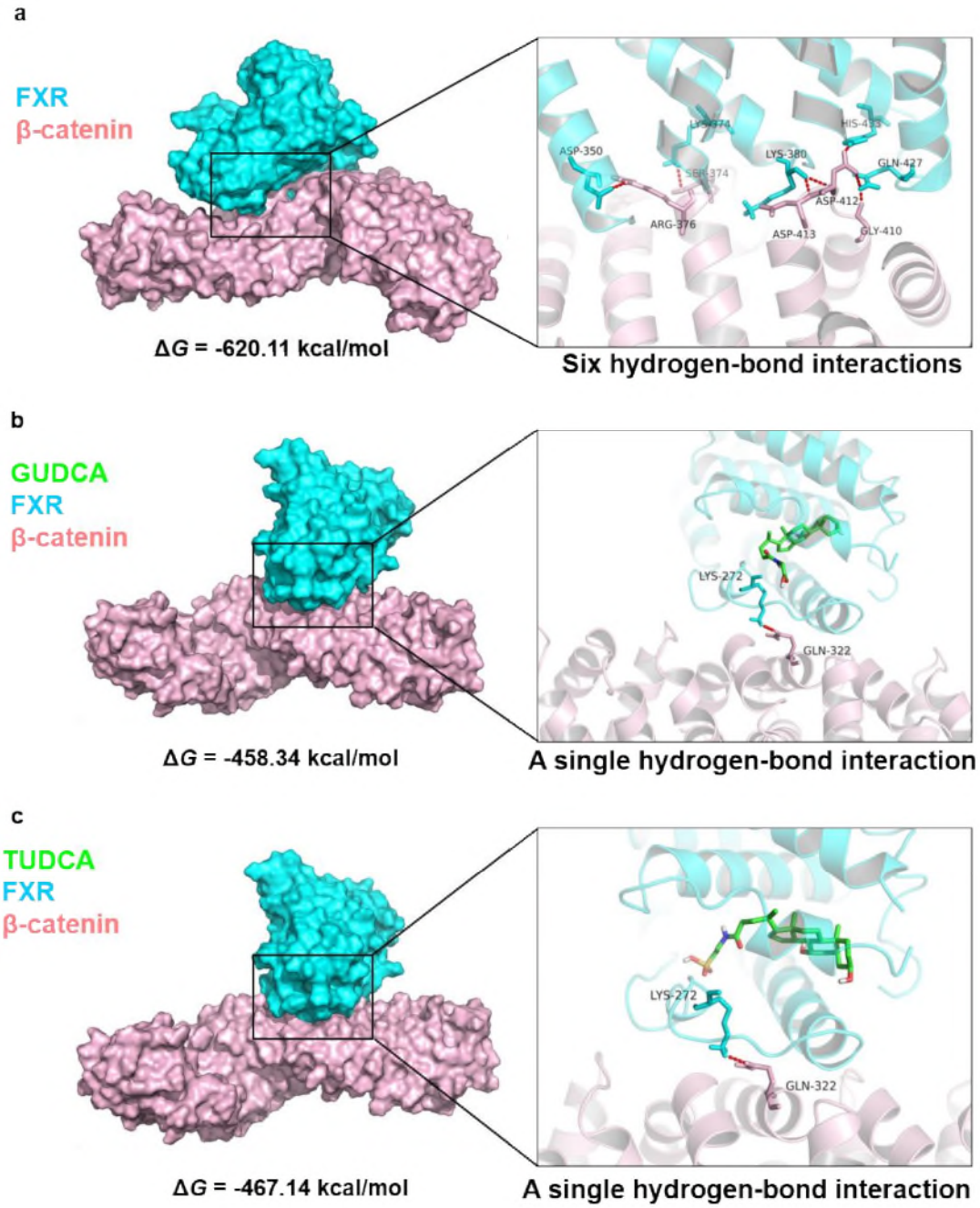
3. While the authors have demonstrated decreased interaction between FXR and β-catenin, it remains unclear if this is the result of decreased FXR expression or some other mechanism. Indeed, the FXR co-IP results seem to show dramatically reduced β-catenin interactions in GBx +AD conditions compared to Sham + AD despite similar pull down of FXR. This suggests that decreased β-catenin interaction with FXR is due to some alternative mechanism, such as conditional interactions dependent on FXR binding to agonistic vs. antagonistic bile acids, however, this was neither tested nor discussed in the manuscript.

Response: We express our sincere appreciation for the insightful comment provided. Nuclear FXR has been reported to interact with β-catenin to form a complex, which subsequently interferes the transcriptional activity of the β-catenin/TCF. Our data showed that the interaction between FXR and β-catenin decreased, and subsequently the β-catenin transcriptional activity was increased after cholecystectomy. On the one hand, GUDCA or TUDCA have been identified as natural antagonists of FXR, which could inhibit the expression level of FXR (***Cell Mol Life Sci. 2022; 79: 527; J Clin Invest. 2020; 130(1): 438 - 450***). And we did observe that FXR expression decreased after GBx, and treatment with GUDCA significantly downregulated FXR expression in intestinal epithelial organoids. Therefore, the decreased interaction between FXR and β-catenin was at least partially attributed to the decreased FXR expression.

On the other hand, the FXR co-IP results seem to show dramatically reduced β-catenin interactions in GBx +AD conditions compared to Sham + AD despite similar pull down of FXR, implying that some alternative mechanism might also contribute to the decreased β-catenin interaction. Previous study showed that TUDCA or GUDCA were molecularly docked with the ligand - binding domain (LBD) of FXR, both were surrounded by amino acid residues like Arg331, Met265, His447 and Tyr369 in this domain ***(Nat Med. 2018; 24(12): 1919-1929)***. It has been reported that FXR can bind to β-catenin through the activation function (AF) surface, disrupting the assembly of

the core β-catenin/TCF4 complex ***(Mol Metab. 2024; 79: 101841).*** Whether FXR binding to agonistic or antagonistic bile acids affects its binding to catenin has not been reported yet. Here, we computationally investigated the direct interaction between FXR and β-catenin by molecular docking (attached below). The FXR/ligand complex structures available in the PDB database were categorized into unliganded states (apo-FXR) and liganded states ***(Comput Struct Biotechnol J. 2021; 19: 2148-2159).*** For the apo-FXR (PDB ID: 5Q0K) and β-catenin (PDB ID: 1JDH), the binding free energy was calculated to be -620.11 kcal/mol, primarily due to the formation of six stable hydrogen bonds through the AF surface (Supplementary Fig. 23a). In contrast, when TUDCA or GUDCA were docked into the antagonistic ligand state of FXR (PDB ID: 4WVD), the binding free energy increased to -458.34 and -467.14 kcal/mol, respectively. This reduction was attributed to a decrease in the number of hydrogen bonds (Supplementary Fig 23b and c), indicating a weaker binding affinity. The conformational changes observed in the AF surface of FXR are likely responsible for different binding affinity. Therefore, the antagonistic bile acids such as TUDCA or GUDCA induced by cholecystectomy may interrupt the interaction between FXR and β-catenin, which might also be a potential mechanism contributing to the decreased binding of FXR and β-catenin.

According to the reviewer's suggestion, we have added the above discussion in the Discussion section of the revised manuscript on Page 21-22.



Supplementary Fig 23. Molecular docking of the interaction between FXR and β-catenin. a Docking of the apo-FXR (PDB: 5Q0K) into the β-catenin (PDB: 1JDH). **b** Docking of the GUDCA/FXR (PDB: 4WVD) into the β-catenin (PDB: 1JDH). **c** Docking of the TUDCA/FXR (PDB: 4WVD) into the β-catenin (PDB: 1JDH). The FXR was colored cyan, and the β-catenin was colored pink. The hydrogen bonds were indicated with red dashed lines. GUDCA, TUDCA and residues contributing hydrogen bonds were highlighted as stick models.

4. In the results section of the main text under the heading “B. breve and R. gnavus control colorectal tumorigenesis through GUDCA production, the authors switch between describing GUDCA in human and TUDCA in mouse without explaining how these two different bile acids relate to each other. This is covered briefly in the discussion section but needs to appear in the results section as part of the logic flow so that the average reader can understand why the authors are presenting the data as it is.

Response: We greatly appreciate the valuable and constructive comment provided. We have added the explanation why we switch between describing GUDCA in human and TUDCA in mouse in the results section to make it more understandable in the revised manuscript on Page 12.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

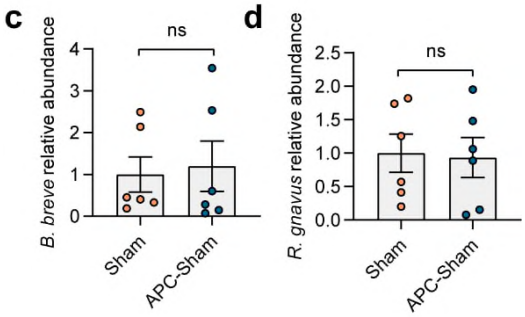
Several issues have been clarified but a number of concerns remain.
The multiplicity of data and the fact that several figures are still not self-explanatory as mentioned in my previous review, make the presentation confusing.
Together with shortcomings, they make the conclusions uncertain.

1. The authors should make clear from the beginning (including the abstract) that the main findings were obtained in two mouse models of CRC. In the setting of these models, cholecystectomy appears to be an aggravating factor of CRC development, and information is lacking regarding dysbiosis and changes in BA metabolism at baseline in these models. These models are very different from patients whose intestine presumably was healthy.

Response: We sincerely appreciate your constructive comments. Regarding the first point, we have revised the abstract and the beginning part of the manuscript to explicitly state that the main findings were derived from two mouse models of CRC.

We have provided a detailed description of the baseline information regarding the changes in gut microbiota and bile acids in these models. The data demonstrated that there was no significant difference in *B. breve* and *R. gnavus* between the two groups at baseline (Supplementary Fig. 8c-d), while the abundance of *B. breve* significantly decreased, and the abundance of *R. gnavus* increased (Fig. 4k-n) after cholecystectomy in these two mice models. Moreover, no significant difference was detected in the fecal TUDCA level between the two groups at baseline (Supplementary Fig. 14d). Cholecystectomy resulted in higher levels of TUDCA in these two mice models (Supplementary Fig. 12c and Supplementary Fig. 14c). Thus, our finding suggests that, in the AOM/DSS and $APC^{min/+}$ models, cholecystectomy led to the changes in specific

species and bile acid. We have added these new data in page 9 and 11 in the revised manuscript.



Supplementary Fig. 8. The gut microbiota diversity in Sham and GBx mice, and microbiota abundance in Sham, APC-Sham, and FMT mouse models, related to Fig. 4. c qPCR analysis of *B. breve* abundance in Sham and APC-Sham groups. **d** qPCR analysis of *R. gnavus* abundance in Sham and APC-Sham groups.

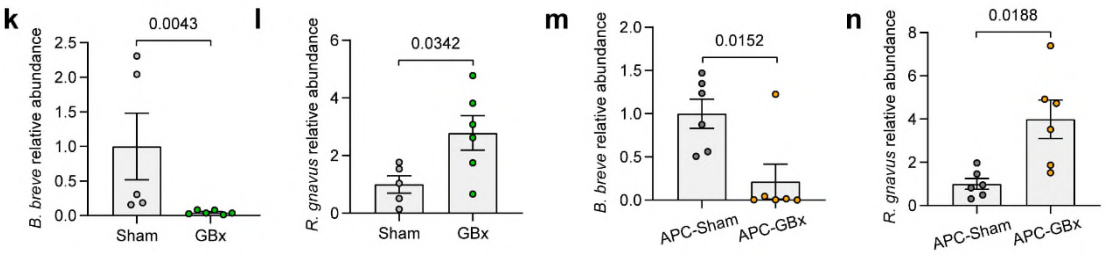
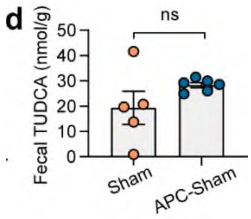
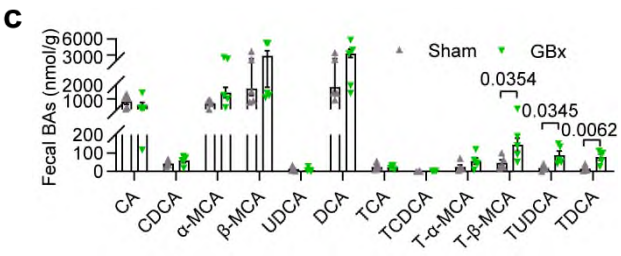


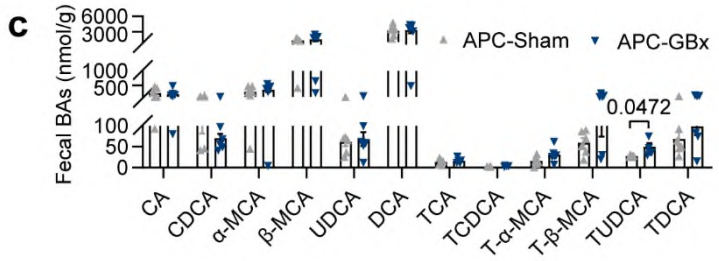
Fig. 4. The gut microbiota profiles in cholecystectomy patients and mice models. k qPCR analysis of *B. breve* abundance in Sham and GBx mice. **l** qPCR analysis of *R. gnavus* in Sham and GBx mice. **m** qPCR analysis of *B. breve* abundance in APC-Sham and APC-GBx mice. **n** qPCR analysis of *R. gnavus* in APC$^{min/+}$mice.



Supplementary Fig. 14. Bile acid profile after cholecystectomy in APC$^{min/+}$ mice, and fecal level of TUDCA between Sham and APC-Sham groups. d Fecal TUDCA level between Sham and APC-Sham groups.



Supplementary Fig. 12. Bile acid profile in mice after cholecystectomy. c Fecal bile acid profiles between Sham and GBx mice.

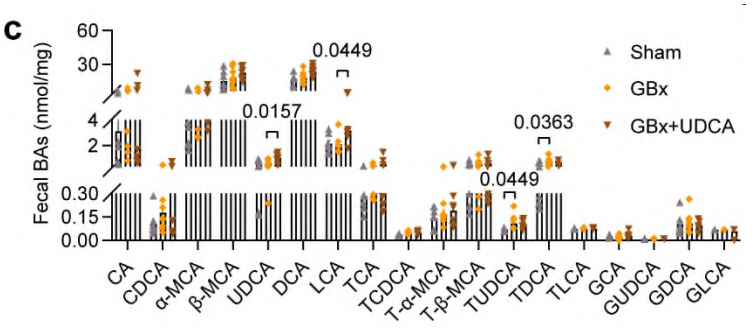


Supplementary Fig. 14. Bile acid profile after cholecystectomy in APC$^{min/+}$ mice, and fecal level of TUDCA between Sham and APC-Sham groups. **c** Fecal bile acid profiles between APC-Sham and APC-GBx groups.

Our mouse models were established to mimic certain aspects of CRC oncogenesis after cholecystectomy. Although they may not fully replicate the human situation, they provide valuable insights into the potential mechanisms. Most importantly, we found that cholecystectomy led to a decrease in the key bacterium *B. breve*, an increase in the amount of *R. gnavus*, and an increase in TUDCA in both cholecystectomy population and cholecystectomized mice, indicating that these mouse models can be used to simulate and explore the possible mechanisms of cholecystectomy-related CRC. We added this explanation in the revised manuscript on page 18-19.

2. Figure 5 is an example of figure, in which the multiplicity of conditions makes it confusing. It raises a major inconsistency : Cholecystectomy (GBx) + R. Gnavus + UDCA causes less CRC than cholecystectomy (h), whereas UDCA gavage should increase GUDCA, and therefor increases CRC (j). The level of TUDCA in UDCA-fed mice was not checked. Typically this figure is not self-explanatory: Abbreviations such as AD, BSH…are not defined; b, c it should be specified if this is fecal BAs; f there is an error in the legend R.gnauvs instead of R. gnavus.

Response: Thank you very much for your comments. To clarify the function of bacterial 7β-hydroxysteroid dehydrogenase (HSDH) activity in GBx-mediated CRC, we carried out an experiment using the 7β-HSDH inhibitor UDCA. Our findings demonstrated that the administration of *R. gnavus* resulted in a rise in the number of tumors, along with an increase in the proportion of adenocarcinoma, high-grade dysplasia, and low-grade dysplasia, while these effects were mitigated by the 7β-HSDH inhibitor UDCA (Fig.5h). Previous studies in both humans and mice have revealed that upon UDCA treatment, lithocholic acid (LCA) becomes the most abundant colonic bile acid modified by intestinal bacteria ***(Acta Pharm Sin B; 2022; 12(5): 2129-2149; Am J Physiol Gastrointest Liver Physiol. 2017; 312(6): G550-G558; Sci Rep. 2020; 10(1): 3895).*** In addition, we have measured the effect of UDCA supplementation on fecal bile acid levels and found that the levels of UDCA and its metabolite LCA increased significantly, while no significant difference was observed in the levels of TUDCA (Supplementary Fig. 18c). Thus, we believe that our results are not contradictory or inconsistent, and we have incorporated these new data on page 14 and included relevant discussions in the "Discussion" section (page 21) of the revised manuscript.

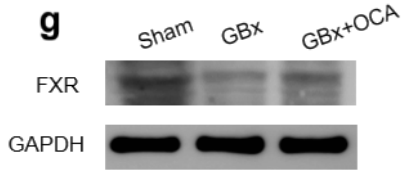


Supplementary Fig. 18. Tumor number and quantitative analysis of H&E staining in mice, as well as the bile acid profile in GBx mice treated with UDCA after AOM/DSS treatment, related to Fig. 5. c Fecal bile acid profiles in GBx mice with or without UDCA treatment.

Finally, we have carefully corrected the details that the reviewer mentioned. All abbreviations such as AD, BSH, etc. have been clearly defined either in the figure legend of the relevant section or in a dedicated abbreviation list. Moreover, we have clearly specified in the figure and figure legend that the data in b and c are derived from fecal bile acids, and corrected the error in the legend of f.

3. Figure 7 is another example of not self-explanatory. Even though the authors propose a mechanism of how GUDCA and TUDCA modify the interaction between FXR and β-catenin, it is not understandable why cholecystectomy has such a dramatic effect on FXR protein expression (Fig. 7e). Likewise, it is difficult to understand why OCA, which is an FXR activator, would have such a dramatic effect on FXR expression (Fig. 7e). Moreover, is not specified either in the Fig. legend or Methods, which part of the intestine was analyzed in Fig. 7e. Was it the ileum or the colon? Was it in tumoral or non-tumoral tissue? FXR is down-regulated in tumoral tissue, as a result of dedifferenciation (not to mistake for a direct effect of BAs).

Response: We appreciate your detailed and constructive comments. In the revised manuscript, we have clearly stated in both the figure legend and the methods section that the colon cancerous tissues were analyzed. Previous research has indicated that OCA is able to activate the FXR ***(J Hepatol. 2016; 64(5): 1158-1166)*** and significantly promotes FXR expression (***Cell. 2019; 176(5): 1098-1112. e18; Biomed Pharmacother. 2017; 96: 1292-1298)***. We observed that FXR expression decreased after GBx, and treatment with OCA significantly upregulated FXR expression in colon cancerous tissues (Fig. 7e). As the reviewer mentioned, we did observe that cholecystectomy had a dramatic effect on FXR expression in colon cancerous tissues, and it is probable that some other effects such as dedifferentiation in tumor tissues contributed to this effect. To validate the effect of cholecystectomy or OCA on FXR expression, we further detected the expression of FXR in the non-tumor tissues of GBx mice treated with or without OCA. Consistently, the results demonstrated that OCA could still reverse the reduction of FXR expression caused by cholecystectomy (Supplementary Fig. 22g). We have diligently incorporated these new findings into the revised manuscript, specifically on pages 17 and 24.



Supplementary Fig. 22. FXR/β-catenin signaling is altered after OCA treatment in GBx mice or GUDCA-treated intestinal organoids, related to Fig.7. g Protein expression of FXR and GAPDH in the colon tissues in each group.

Previous studies revealed that GUDCA or TUDCA were identified as natural antagonists of FXR, which could inhibit the expression level of FXR ***(Cell Mol Life Sci. 2022; 79: 527; J Clin Invest. 2020; 130(1): 438 - 450).*** We did observe that FXR expression decreased after GBx, and treatment with GUDCA significantly downregulated FXR expression. Thus, it is believed that cholecystectomy led to an increase in the levels of the FXR antagonists TUDCA or GUDCA, which in turn inhibited FXR expression and this inhibition could be reversed by OCA.

4. The shift between the 2 models of CRC APCmin/+ vs AOM/DSS is not justified and also difficult to follow.

Response: We are extremely grateful for your comment regarding the shift between the $APC^{min/+}$ and AOM/DSS CRC models in our study. Human colorectal cancer arises from the intricate interplay of both genetic and environmental factors. The $APC^{min/+}$ model predominantly serves to simulate genetically-driven CRC. In contrast, the AOM/DSS model operates through chemical induction. Our findings indicated that, in both mouse models, cholecystectomy led to a decrease in the abundance of *B. breve* and an increase in the abundance of *R. gnavus* (Fig.4i-n). Moreover, we observed higher levels of TUDCA in both mouse models following cholecystectomy (Supplementary Fig. 13c, 14c). This finding suggests that in both the AOM/DSS and the $APC^{min/+}$ models, the changes in key bacteria and bile acids induced by cholecystectomy are consistent. More importantly, we observed that cholecystectomy exacerbated early and late colitis in mice (Fig. 2), and colorectal tumorigenesis developed much earlier after cholecystectomy (Fig. S3). Since the AOM/DSS model places particular emphasis on inflammation-related carcinogenesis, we mainly selected this model for subsequent studies. We have added the above explanations in the discussion section to make the shift more comprehensible. Specifically, these additions can be found on page 18-19.

5. Key informations regarding Materials and methods, which were previously requested, such as which part of the intestine was analyzed cannot be provided only as supplementary especially as so little details are provided in Fig. legends.

Response: We appreciate your detailed and constructive comments. We have added the key information in the relevant figure legends to make it more understandable.

6. The abstract is also poorly informative regarding the methods used.

Response: We have added more information regarding the methods in the abstract, which has been highlighted in red in the abstract.

Additional comments

7. Table S1: change health for healthy; NAFLD for MAFLD (to define)

The authors wrote: "Importantly, previous studies showed that the occurrence rate of CRC increased markedly after cholecystectomy (Br J Cancer. 2003; 88: 79-83; PLoS One. 2017; 12: e0181852; Front Med. 2022; 9: 1000563). Therefore, integrating the previous literature and our findings suggests that the risk of CRC occurrence increases after cholecystectomy."

I would delete markedly and change the 2nd sentence, for "However, the difference we found in the incidence of CRC between cholecystectomized and healthy subjects was no significant, which could be explained by a small sample size."

Response: In the revised Table S1, we have changed the word "health" to "healthy". Meanwhile, we have changed "NAFLD" into "MAFLD" and have defined "MAFLD" in the revised manuscript.

Regarding the suggestions for the text, we have deleted the word "markedly" and changed the 2nd sentence as "However, the difference we found in the incidence of CRC between cholecystectomized and healthy subjects was not significant, which could be explained by a small sample size".

8. Fig. S16: a. please define all abbreviations; b. color error of one dot (orange instead

of black) to correct in the GUDCA group; c. why the statistical test was different in b and c? e. please show dysplastic mucosae and carcinoma by different arrows.

Response: In the revised manuscript, all the abbreviations have been clearly defined in the figure legend. Moreover, the erroneously colored orange dot in Fig. S16b has been rectified to black. Regarding the statistical tests, in Fig. S16b, the two datasets did not exhibit a normal distribution. Consequently, non-parametric statistical methods were employed. Conversely, in Fig. S16c, the two datasets adhered to a normal distribution; thus, parametric statistical methods were utilized. Finally, we have used different arrows to show dysplastic mucosae and carcinoma respectively in Fig. S16e.

9. Fig. 4. The legends of g. and h. are not consistent

Response: In the revised manuscript, we have revised the legends for Fig. 4g and Fig. 4h to maintain their consistency.

10. There are still a number of syntax errors, e.g. lines 82-83, “significantly difference”, lines 162-163, “To deeply explore…We…”; lines 256-257, “As conjugated…We previously…”; line 285, “…inhibitors UDCA…”

Response: We are extremely grateful for your comments and suggestions. Regarding the specific errors you mentioned: In lines 82 - 83, the phrase ″significantly difference″ has been rectified to″significant difference″. For lines 162 - 163, the construction “To deeply explore…We…” has been amended to “To deeply explore…we…”. In lines 256 - 257, “As conjugated…We previously…” has been adjusted to “As conjugated…we previously…”; in line 285, “…inhibitors UDCA…” has been revised to “the 7β-HSDH inhibitor UDCA”.

In addition to addressing the issues pointed out by the reviewers, we have conducted a complete grammar check throughout the manuscript to avoid syntax errors in the revised version.

Reviewer #3 (Remarks to the Author):

The authors have performed key experiments that adequately address this reviewer's concerns. The manuscript is now suitable for publication.

Response: We are truly grateful to receive the positive feedback.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors carefully addressed my comments and clarifications have been provided. Yet, the conclusions are overstated. Colorectal carcinogenesis was exacerbated or accelerated in the mouse models rather than promoted as indicated in the results and in the title. Cholecystectomy exacerbates or accelerates colorectal carcinogenesis would be more appropriate. The mechanisms also remain questionable. Figure 8 indicates that the effect of cholecystectomy on colorectal carcinogenesis is mediated by GUDCA but GUDCA was not increased in cholecystectomized mice. It was increased in cholecystectomized humans who did not develop significantly more colorectal cancer.

Response: We sincerely appreciate the constructive comments. We have revised the expressions in the results section, and used “exacerbates” to replace “promotes”. And we also changed the manuscript title into "Cholecystectomy-related gut microbiota dysbiosis exacerbates colorectal tumorigenesis". These updates can be found on page 1, 2, 4, 6, 18, 25, 46, 47 and 48.

Actually, in our mouse model, we observed that both GUDCA and TUDCA could exacerbate colorectal carcinogenesis. Since TUDCA was the predominant form in mice, we have revised the mechanism diagram by replacing GUDCA with TUDCA in both Figure 8 and its corresponding legend.

Minor comment: In the discussion, lines 400-401 “Given that the AOM/DSS model places great emphasis on inflammation-related carcinogenesis, this model was used for the following study”. What does Following refers to?

Response: Thank you for this meticulous question. To enhance clarity, we have revised the sentence to: “Given that the AOM/DSS model places great emphasis on inflammation-related carcinogenesis, we employed this model in the following study, which involved fecal microbiota transplantation, single bacterial colonization, and bile acid supplementation”.