様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

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研究課題名（和文）Periodontitis induced alterations of gut microbial metabolite promotes liver cancer through senescence

研究課題名（英文）Periodontitis induced alterations of gut microbial metabolite promotes liver cancer through senescence

研究代表者
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交付決定額（研究期間全体）：（直接経費）　　3,200,000円

研究成果の概要（和文）：本研究は、肝臓の肝星細胞に対するDCAの効果、およびそれに続く肝癌細胞（HCCs）に対する間接的影響を検証する。ヒトの肝臓の腺腫細胞を、DCAを作用させたLX2細胞（肝星細胞）培養上清で培養した。結果として、DCAはLX2の細胞老化を引き起こすことで、細胞周期の停止に伴う増殖抑制とSASP 因子の誘導を認めた。DCAを作用させたLX2細胞の培養上清でHCCsを培養すると、EMT誘導による遊走能および浸潤能の促進を認めた。これらの変化はIL-8あるいはTGF-βに対する中和抗体を作用させることで消失した。HCC患者検体の病理学的検索を行うと、老化したHSCsをHCC周囲の間質にて認めた。

研究成果の学術的意義や社会的意義
Our study provided the new insights into the molecular genetics of liver cancer under effects of Deoxycholic acid. In addition, we proposed a novel therapeutic strategy against HCC development, including interventions targeting senescent HSCs and SASP factors, particularly IL8 and TGFβ.

研究成果の概要（英文）：This study aimed to examine the effect of DCA on hepatic stellate cells (HSCs), a major component of nonparenchymal cells in the liver, and its subsequent indirect effect on HCC cells. LX2 cells, a human HSC line, were treated with DCA in vitro. Then, HuH7 cells, a human hepatoma cell line, were incubated in conditioned media of DCA-treated LX2 to investigate the subsequent effect focusing on malignant behaviors. DCA resulted in cellular senescence in LX2 with the decreased cell proliferation via cell cycle and the induction of senescence-associated secretory phenotype (SASP) factors. HCC cells were treated with DCA treated LX2-conditioned media, that promoted cell migration and invasion via induction of epithelial mesenchymal transition. These changes were attenuated in the presence of neutralizing antibody against IL8 or TGFb. Pathological analysis of surgical specimens from HCC patients revealed that senescent HSCs were detected in the stroma surrounding HCC.

研究分野：Oncology

キーワード：Liver　metabolism　bile acids　Senescence　Invasion　cancer

様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９（共通）

１．研究開始当初の背景

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related deaths worldwide. Although there have been many advances in diagnosis and treatment for HCC, prognosis of patients with HCC remains poor, with overall 5-year survival rate of 18%. Hence, the elucidation of HCC pathogenesis remains one of critical issues.Several evidences demonstrate the involvement of bile acids in liver tumorigenesis in human and animal models, and suggest the possibility that DCA involves in carcinogenesis and tumor progression of HCC. However, its underlying mechanisms remain not fully understood. Salivary microbiology may alter gut bacterial composition, thereby increasing the level of secondary bile acid deoxycholic acid (DCA), a gut bacterial metabolite known to cause DNA damage, in enterohepatic circulation that causes the increase of DCA in liver. The elevated of DCA stimulates senescence-associated secretory phenotypes (SASP) in hepatic stellate cells (HSCs), which accelerate the secretion of various inflammatory and tumor promoting factors in the liver.

２．研究の目的

The aim of this study is to clarify that the increased level of DCA produced by periodontopathic bacteria mediated gut bacteria alterations promote periodontitis-associated hepatocellular carcinoma and underlying molecular mechanism *in vitro* and *in vivo*.

３．研究の方法

a. Set up a system to induce liver cancer in mice

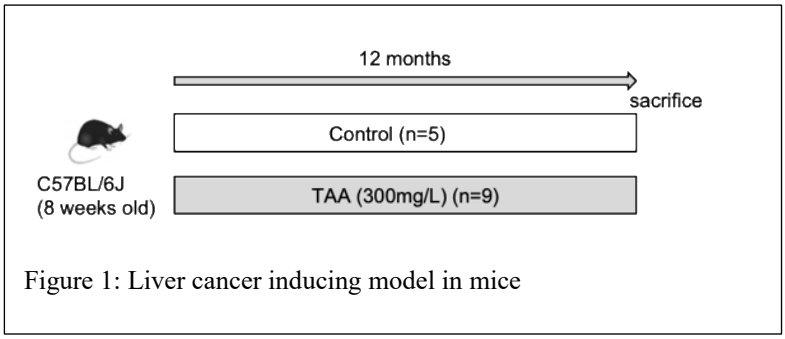

Figure 1: Liver cancer inducing model in mice

b. Investigate cellular senescence in HSCs

We performed Sudan Black B staining, immunohistochemical staining SASP markers of liver cancer section.

c. Examine whether DCA has the key roles in HCC development

We performed immunohistochemistry of DCA in liver cancer section

d. Examine whether bile acids, particularly DCA treatment induces SASP in vitro

To further support and extend our murine data to human biology, we tested whether bile acids, particularly DCA treatment can induce SASP in hepatic stellate cells.

e. Observe the signs of cellular senescence and SASP in human HCC samples

We performed Sudan Black B staining, immunohistochemical staining SASP markers of liver cancer section and analyzed in TCGA database

４．研究成果

DCA suppressed cell growth with cell cycle arrest in HSCs

First, we examined the effect of DCA on the cell growth of HSCs. DCA significantly reduced the growth of LX2 cells in a dose-dependent manner compared to vehicle-treated cells. Consistently, we found that DCA-treated LX2 cells showed a marked increase in the number of Ser139-phosphorylated histone H2AX (γH2AX) foci as an indicator of DNA

damage (DNA double-strand breaks) (Fig. 2). In addition, we examined the cell cycle distribution of LX2 cells after 7 days of DCA treatment using propidium iodide staining. The percentage of cells in G0/G1 phase was significantly increased in DCA-treated cells compared to the control cells. Furthermore, the expression levels of p21 and p53 were upregulated at mRNA and protein level by the treatment of DCA (Fig. 3).

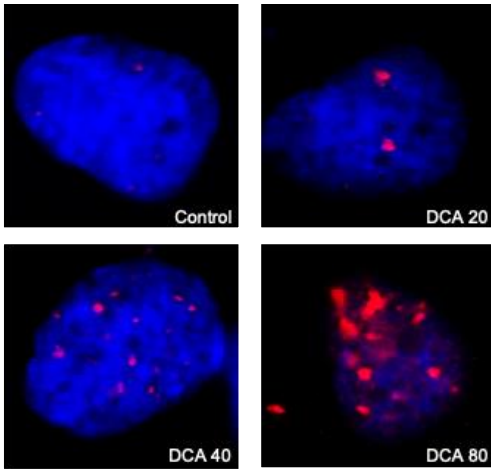

Figure 2: DCA treatment induced an impaired DNA damage response in LX2 cells

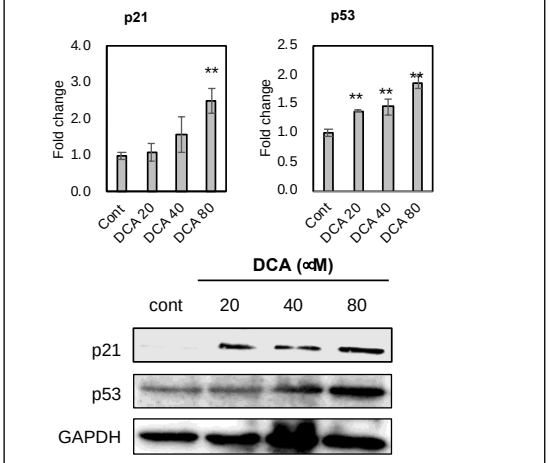

Figure 3: The expression of p21 and p53 in LX2 under DCA treatment

DCA induced cellular senescence in HSCs

As p53/p21 pathway is involved in cellular senescence process, we stained LX2 cells with SA-β-gal, a senescence-associated marker, and performed ELISA assay to quantify the level of SA-β-gal. The results showed a gradual increase in positively stained cells for each DCA concentration, and the difference reached statistical significance at the concentration of 80 μM as quantified by ELISA (Fig. 4). We also found significant increases of IL1β, IL6, IL8, Groα, TGFβ and MMPs transcriptions, which are well known as SASP components, in LX2 cells with DCA treatment for 7 days (Fig. 5) as well as the increases of IL6, IL8 and TGFβ secretion in concentrated condition media of LX2 with DCA treatment. The analysis of signaling pathway demonstrated the significant increase in phosphorylated p65 in response to DCA. In contrast, the degree of ERK phosphorylation, a major regulator of cell proliferation was decreased. Taken together, these data suggest that DCA promotes the acquisition of senescence in HSCs, and consequent secretion of SASP components.

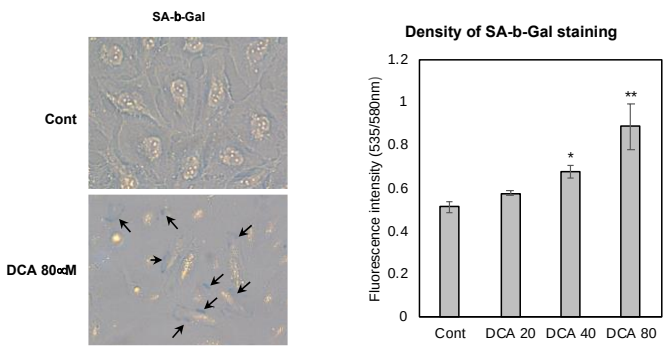

Figure 4: SA-b-Gal increased in LX2 under DCA treatment

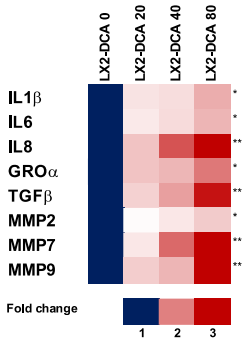

Figure 5: DCA upregulated SASP in LX2 cells

CM of senescent LX2 cells affected biological characteristics of HCC cells

To investigate the effect of SASP factors secreted by DCA-treated HSCs on HCC cells, we performed *in vitro* migration and invasion assays by culturing HuH7 cells in CM. On day 2, the migration and invasion activities of HuH7 cells cultured in CM from DCA-treated LX2 cells (CM-LX2-DCA) was significantly higher than those in CM of vehicle-treated LX2 cells (CM-LX2), and HuH7 cells cultured in CM-LX2-DCA showed more spindle-like mesenchymal morphology and lacked the epithelial hallmarks of HCC cells. Since EMT regulates cancer invasion and metastasis, we assessed whether EMT was induced in HCC cells cultured in CM from LX2 cells. As expected, the gene expression of EMT markers, including N-cadherin, Snail, and Vimentin were significantly increased in HuH7, while the expression of E-cadherin was reduced (Fig. 6). In addition, we also found that MMP1, MMP7, MMP9 and MMP13 were upregulated in HuH7 cells cultured in CM-LX2-DCA (Fig. 6). Importantly, there was no direct effect of DCA on the expression of EMT markers in HuH7, excluding the possibility that residual DCA in CM induced EMT (Fig. 6). These data suggest that the mediators secreted by HSCs in response DCA promotes cell invasion and migration of HCC cells via EMT and MMPs activation.

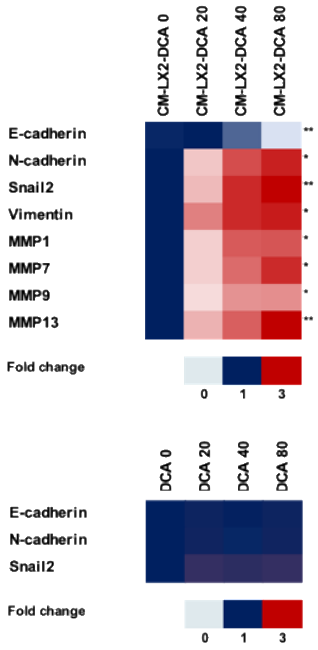

Figure 6: Heatmap of qRT-PCR analysis of E-cadherin, N-cadherin, and Snail2 and MMPs expression in HuH7 cells cultured with or without DCA.

TGFβ and IL8 are the major contributors on the malignant behaviors of HCC

Among SASP components investigated in this study, gene expressions of IL1β, IL6, IL8, GROα, and TGFβ were significantly upregulated in LX2 cells in response to DCA. Therefore, we speculated these mediators were the major factors that promote the migration and invasion of HCC cells. Hence, CM of LX2 cells with 80 μM of DCA treatment was utilized for migration and invasion assay in the presence of neutralizing monoclonal antibodies against these mediators. Interestingly, the addition of TGFβ- and IL8-neutralizing antibodies significantly inhibited the migration and invasion of HuH7 cells cultured in CM-LX2-DCA while the other mediators did not exhibit suppressive effect. In addition, TGFβ- and IL8-neutralizing antibodies remarkably attenuated gene induction of N-cadherin and Snail2 in HuH7 cultured in CM-LX2-DCA. Conversely, there was clearly no obvious effects by the concomitant treatment of neutralizing antibody against IL6, IL1β, and GROα.

Upregulation of SASP components and EMT markers in human HCC samples.

We analyzed an HCC cohort from the TCGA database (https://xenabrowser.net/) to determine the expression of genes associated with SASP components. As shown in Fig. 7, the expression pattern of SASP components (IL1β, IL6, IL8, Groα, TGFβ, and MMPs) were significantly correlated with that of vimentin, which is well known as a marker of invasion and metastasis in HCC. Interestingly, these findings were accompanied by upregulation of αSMA (ACTA2), COL1A1, LOX, reliable markers of HSCs. We next evaluated the association between gene expression of SASP factors and patient prognosis using Kaplan-Meier method. Based on our observations of migration and invasion assays with neutralizing antibodies (Fig.

8), we speculated that IL8 and TGFβ are predominantly associated poor survival. Unexpectedly, in addition to IL8 and TGFβ, high expressions of IL1β, GROα, MMP7, and MMP9 were also correlated with poor prognosis (Fig. 8), suggesting the possibility that these mediators promote the malignant progression of HCC with no impact on cell migration and invasion.

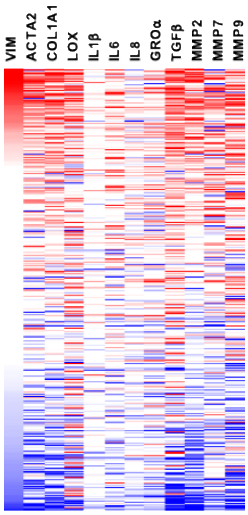

Figure 7: Heat map of genes associated with the SASP and HSCs in HCC patients

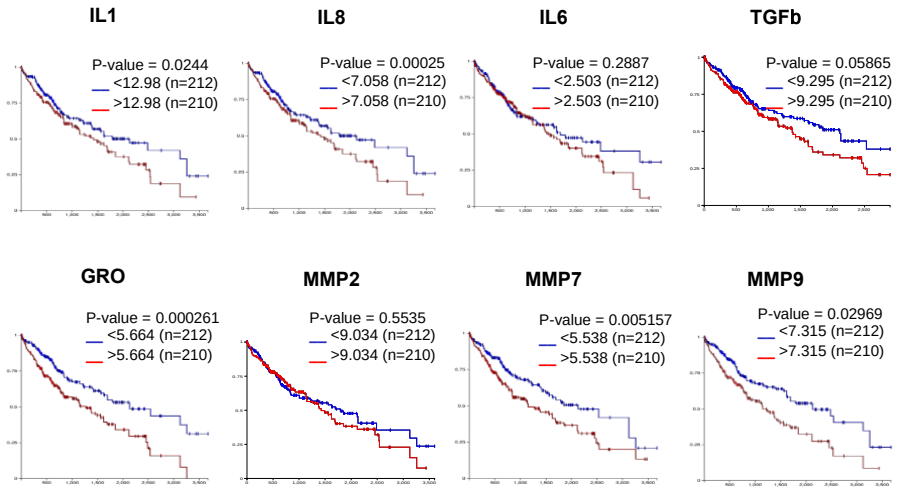

Figure 8: Kaplan-Meier analysis of survival in HCC patients according to SASP gene expression

We next determined the presence of senescent fibroblast cells surrounding HCC by SBB staining in Fig. 9 (upper two panels). Together with these findings, E-cadherin expression was preserved in the noncancerous cells but reduced in cancer cells, whereas the expression of N-cadherin and vimentin was enhanced in cancer cells nearby stromal tissue (Fig. 9, lower two panels), suggesting that EMT occurred at the invasive border where senescent HSCs are present.

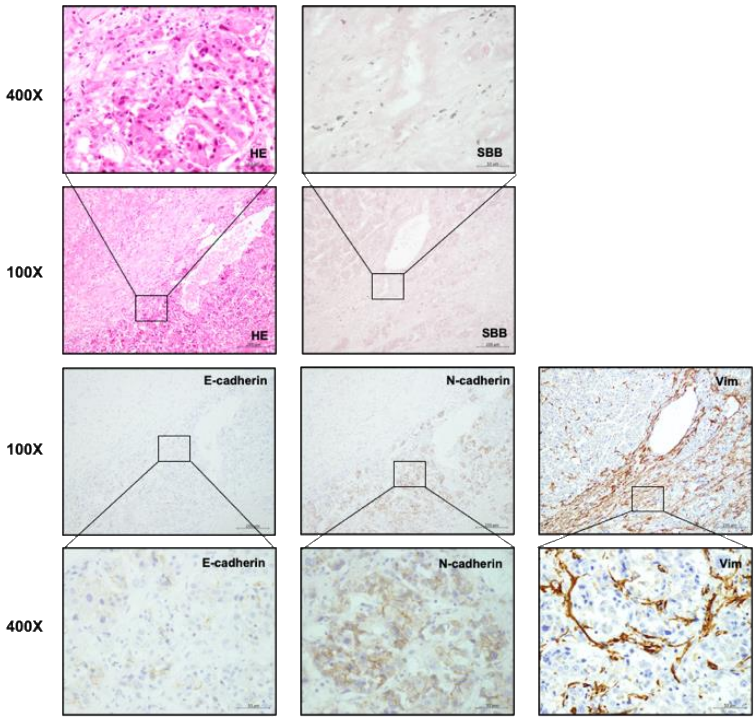

Figure 9: HE staining; IHC analysis of E-cadherin, N-cadherin and vimentin expression; and SBB staining at the invasive border of the tumor in clinical samples of HCC. SBB, Sudan Black B.

５．主な発表論文等

〔雑誌論文〕　計6件（うち査読付論文　4件／うち国際共著　4件／うちオープンアクセス　0件）

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２．論文標題 Deoxycholic Acid-Induced Senescent Hepatic Stellate Cells Promote Malignant Behaviors of Hepatocellular Carcinoma Cells: Implication to Underlying Mechanisms of Metabolic Syndrome-Based Fatty Liver and Carcinogenesis	５．発行年 2019年
３．雑誌名 Gastroenterology	６．最初と最後の頁 S-769
掲載論文のDOI（デジタルオブジェクト識別子） 10.1016/S0016-5085(19)38870-5	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 該当する

１．著者名 Tomoki Kobayashi, Keishi Kanno, Phuong Thao Nguyen, Akiko Sugiyama, Yuichiro Otani, Nobusuke Kishikawa and Susumu Tazuma	４．巻 70
２．論文標題 Molecular Mechanisms in which Periostin Induces Hepatic Steatosis In Vivo and In Vitro	５．発行年 2019年
３．雑誌名 Hepatology	６．最初と最後の頁 1286
掲載論文のDOI（デジタルオブジェクト識別子） 10.1002/hep.30941	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 該当する

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２．論文標題 Periostin antisense oligonucleotide prevents hepatic steatosis and fibrosis in a mouse model	５．発行年 2020年
３．雑誌名 Journal of Gastroenterology and Hepatology	６．最初と最後の頁 1~11
掲載論文のDOI（デジタルオブジェクト識別子） 10.1111/jgh.15088	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 該当する

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2．論文標題 Periostin antisense oligonucleotide prevents hepatic steatosis and fibrosis in a mouse model of non‐alcoholic steatohepatitis	5．発行年 2020年
3．雑誌名 Journal of Gastroenterology and Hepatology	6．最初と最後の頁 2140～2150
掲載論文のDOI（デジタルオブジェクト識別子） 10.1111/jgh.15088	査読の有無 無
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1．著者名 Nguyen Phuong Thao、Kanno Keishi、Pham Quoc Thang、Kikuchi Yuka、Kakimoto Masaki、Kobayashi Tomoki、Otani Yuichiro、Kishikawa Nobusuke、Miyauchi Mutsumi、Arihiro Koji、Ito Masanori、Tazuma Susumu	4．巻 146
2．論文標題 Senescent hepatic stellate cells caused by deoxycholic acid modulates malignant behavior of hepatocellular carcinoma	5．発行年 2020年
3．雑誌名 Journal of Cancer Research and Clinical Oncology	6．最初と最後の頁 3255～3268
掲載論文のDOI（デジタルオブジェクト識別子） 10.1007/s00432-020-03374-9	査読の有無 無
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1．発表者名 Nguyen Thao P.、Kanno Keishi、Kobayashi Tomoki、Pham Thang、Tazuma Susumu
2．発表標題 Deoxycholic acid-Induced senescent hepatic stellate cell promote malignant behaviors of hepatocellular carcinogenesis
3．学会等名 120th Annual Meeting, American Gastroenterological Association（国際学会）
4．発表年 2019年

1．発表者名 Phuong Thao Nguyen
2．発表標題 Deoxycholic acid-induced senescent hepatic stellate cells promote invasion and metastasis of hepatocellular carcinoma cells
3．学会等名 Japanese Bile Acid meeting
4．発表年 2019年

１．発表者名
Tomoki Kobayashi, Keishi Kanno, Phuong Thao Nguyen, Akiko Sugiyama, Yuichiro Otani, Nobusuke Kishikawa and Susumu Tazuma

２．発表標題
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２．発表標題
Contributions of hepatic stellate cells into hepatocellular carcinoma

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Nguyen PT, Kanno K, Pham QT, Kobayashi T, Miyauchi M, Arihiro K, Tazuma S, Ito M.

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Senescent hepatic stellate cells caused by deoxycholic acid modulates malignant behavior of hepatocellular carcinoma.

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The 43rd annual meeting of The molecular Biology Society

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2020年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関