Title 論文題目	MicroRNA-31 reflects IL-6 expression in cancer tissue and is related to poor prognosis in cholangiocarcinoma. (胆道癌において microRNA-31 は癌組織の IL-6 発現を反映し不良な予後と相関する)
Author(s) 著　者	石上，敬介
Degree number 学位記番号	甲第 3027 号
Degree name 学位の種別	博士（医学）
Issue Date 学位取得年月日	2018-03-31
Original Article 原著論文	札幌医学雑誌　第 87 巻 1 号　平成 31 年 1 月　掲載予定
Doc URL	
DOI	
Resource Version	Author Edition

Original Article

MicroRNA-31 reflects IL-6 expression in cancer tissue and is related with poor prognosis in cholangiocarcinoma.

Running head: IL-6 and miR-31 in cholangiocarcinoma

Keisuke Ishigami[1], Katsuhiko Nosho[1], Hideyuki Koide[1], Shinichi Kanno[1], Kei Mitsuhashi[1], Hisayoshi Igarashi[1], Masahiro Shitani[1], Masayo Motoya[1], Yasutoshi Kimura[2], Tadashi Hasegawa[3], Hiroyuki Kaneto[4], Ichiro Takemasa[2], Hiroshi Nakase[1]

[1] Department of Gastroenterology and Hepatology, Sapporo Medical University School of Medicine, Sapporo, Japan

[2] Department of Surgery, Surgical Oncology and Science, Sapporo Medical University School of Medicine, Sapporo, Japan

[3] Department of Surgical Pathology, Sapporo Medical University School of Medicine, Sapporo, Japan

[4] Department of Gastroenterology, Muroran City General Hospital, Sapporo, Japan

Correspondence to:
Katsuhiko Nosho M.D., Ph.D.
Department of Gastroenterology and Hepatology, Sapporo Medical University School of Medicine
S-1, W-16, Chuo-ku, Sapporo, 060-8543 Japan
Tel: +81 (11) 611-2111
Fax: +81 (11) 611-2282
E-mail: nosho@sapmed.ac.jp

Word count: 2670

Abbreviations: CI, confidence interval; FFPE, formalin-fixed paraffin-embedded; HR, hazard ratio; IL-6, Interleukin-6; miR-31, microRNA-31; OS, overall survival; PDAC, pancreatic ductal adenocarcinoma; qRT-PCR, quantitative reverse transcription-PCR; SD, standard deviation; SOCS3, suppressor of cytokine signalling 3; STAT3, Signal Transducers and Activator of Transcription 3; UICC, Union for International Cancer Control

Abstract

Cholangiocarcinoma is a highly aggressive malignancy wherein early diagnosis is difficult and few treatment options are available. MicroRNA-31 (miR-31) is reported to be related with survival in patients with gastrointestinal cancers; however, the regulatory mechanism of miR-31 and association between miR-31 expression and survival in patients with cholangiocarcinoma cases have not been established. Thus, we evaluated miR-31 expression in cholangiocarcinoma tissues and assessed its relationship with prognosis. Additionally, we examined the effects of several cytokines on miR-31 expression. The study included 81 samples of cholangiocarcinoma tissues. MiR-31 expression in cholangiocarcinoma cells was significantly higher than that in normal bile duct epithelial cells ($P = 0.038$). There were no significant associations between miR-31 expression and clinical or pathological characteristics, except for tumour size ($P = 0.012$). In Kaplan–Meier analysis, high miR-31 expression was significantly associated with shorter survival (log-rank test, $P = 0.0082$). In multivariate Cox regression analysis, high miR-31 expression was significantly associated with prognosis ($P = 0.043$), independent of clinical or pathological features. IL-6 significantly promoted miR-31 expression and cell proliferation in a dose-dependent manner, and the inhibition of STAT-3 signalling significantly suppressed miR-31 expression and cell proliferation. In conclusion, high miR-31 expression was observed in cholangiocarcinoma cells, and this high expression was significantly associated with poor prognosis in patients. The IL-6-STAT-3 signalling pathway regulated cholangiocarcinoma cell proliferation and miR-31 expression. Our findings suggest that miR-31 may be a promising biomarker that reflects IL-6 expression in cholangiocarcinoma tissues and predicts poor prognosis.

Introduction

Cholangiocarcinoma is a highly aggressive malignancy that arises from bile duct epithelial cells. Surgical treatment is considered to be the only curative treatment for a cholangiocarcinoma; however, several patients with cholangiocarcinoma are diagnosed only at an advanced stage because of the difficulty in early diagnosis. In recent years, the overall survival rate and therapeutic options for cholangiocarcinoma have not improved. Therefore, better understanding of the pathogenesis of cholangiocarcinomas is urgently required.[1-3]

MicroRNAs are a class of small non-coding RNA molecules that function as post-transcriptional gene regulators, and they have been increasingly recognised as useful biomarkers in various human cancers.[4-8] A recent study showed that microRNAs can act as both oncogenic and tumour suppressive agents, depending on the genes they regulate.[9, 10] We previously reported the negative association between miR-31 expression in cancer tissues and survival among patients with gastrointestinal cancers, including colorectal and pancreatic cancers.[11-13] With regard to cholangiocarcinoma, miR-31 is reported to be upregulated in tumour tissues, and it not only promotes cellular proliferation but also inhibits apoptosis.[14-16] However, no study has reported the association between miR-31 expression and survival among patients with cholangiocarcinoma.

Previous reports have demonstrated that chronic inflammation can affect carcinogenesis and that a large part of the immune system depends on cytokines. Interleukin-6 (IL-6), one of the most common inflammatory cytokines, is produced by diverse cell types and is a crucial mediator of immunity. IL-6 receptors transduce signals via gp130 (also known as IL-6Rβ), which is a shared receptor chain of the IL-6 family and is a strong inducer of Signal Transducers and Activator of Transcription 3 (STAT3) activation. IL-6 plays several important roles in cancer progression, and it drives processes, including proliferation, migration and angiogenesis. IL-6-dependent

STAT3 signalling is a critical promoter of cancer cell proliferation and survival.[17-20] In addition, the gene locus of *miR-31* has been reported to possess a STAT-3 binding site; therefore, IL-6 was found to induce miR-31 expression in vitro.[21, 22] However, these relationships have not been established in a gastrointestinal cancer model, including a cholangiocarcinoma model.

Thus, we evaluated miR-31 expression in cholangiocarcinoma tissues and assessed its relationship with prognosis. Furthermore, we examined the effects of several cytokines on miR-31 expression in cholangiocarcinoma cells to determine whether it could reflect cytokine expression in cholangiocarcinoma tissue.

Materials and Methods

Patients and tissue specimens

Formalin-fixed, paraffin-embedded (FFPE) tissues of 81 patients with cholangiocarcinomas (stages I–IV, in accordance with Union according to International Cancer Control (UICC) 7th edition), who underwent surgical treatment at Sapporo Medical University Hospital and Muroran City General Hospital between March 2001 and September 2015, were collected. To avoid selection bias as much as possible, the FFPE tissue specimens were collected consecutively. Cholangiocarcinoma was classified by location as follows: intrahepatic cholangiocarcinoma (IHCCa, n = 16), extrahepatic cholangiocarcinoma (EHCCa, n = 37) and gallbladder cancer (GBCa, n = 28). Patients were followed until death or December 2015 (whichever was first). Informed consent was obtained from all the patients before specimen collection. This study was approved by the respective institutional review boards of the participating institutions.

RNA extraction from cholangiocarcinoma tissue and quantitative reverse transcription-PCR of microRNA-31

We have collected the cholangiocarcinoma cells and adjacent normal bile duct epithelium cells from FFPE samples of cholangiocarcinoma patients using the MMI Cellcut plus microdissection system (MMI, Eching, Germany). Total RNA was extracted using miRNeasy FFPE Kit (Qiagen, Valencia, CA, USA). MicroRNA-31 (miR-31)-5p expression was analysed by quantitative reverse transcription-PCR (qRT-PCR) using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and TaqMan microRNA Assays (Applied Biosystems) as previously described.[11] U6 snRNA (RNU6B; Applied Biosystems)

served as an endogenous control. MiR-31 expression was calculated using the equation $2^{-\Delta CT}$, where $\Delta C_T = (C_T$ miR-31 - C_T U6). To calculate the relative expression of miR-31 in each cholangiocarcinoma, $2^{-\Delta CT}$ of cancer tissue was divided by $2^{-\Delta CT}$ of normal tissue, as previously described.[11]

Cholangiocarcinoma cell lines and induction of miR-31 expression

In this study, we used two cholangiocarcinoma cell lines (HuCCT-1 and TFK-1). HuCCT-1 was provided by HSRRB, and TFK-1 was provided by RIKEN BRC. Each cell lines were validated by analysis of STRs (GenePrint® 10 System, Promega, Barcelona, Spain). Cells were grown in RPMI-1640 (Sigma-Aldrich, St. Luis, MI, USA) supplemented with heat-inactivated 10% FBS (Sigma-Aldrich) in an atmosphere of 5% CO2 at 37°C. Cells showing 70% confluency were stimulated with 0 $ngmL^{-1}$, 20 $ngmL^{-1}$ and 50 $ngmL^{-1}$ of IL-6 (PEPROTECH, Rock Hill, NJ, USA), TNF-α (PEPROTECH), IL-1β (PEPROTECH), IL-17A (PEPROTECH) and IL-10 (PEPROTECH). Cells were harvested for qRT-PCR 24 h after stimulation. To verify the involvement of STAT-3, each cell line was also treated with S3I-201 (Sigma-Aldrich) for 24 h at final concentration of 30 μM or 300 μM. In the study involving S3I-201, each sample contained an equal amount of DMSO to exclude the possibility of the effects of DMSO.

Western blotting

To confirm the effect of S3I-201, we harvested cells after 24 h of treatment with S3I-201 at final concentration of 30 μM, 100 μM or 300 μM. Cultured cholangiocarcinoma cells were lysed in Cell lytic-M (Sigma-Aldrich) containing PhosSTOP (Sigma-Aldrich) and Complete mini (Sigma-Aldrich). Antibodies used for western blotting were as follows: rabbit anti-STAT3

(phosphor Y705) antibody (Abcam, Cambridge, UK), rabbit anti-STAT3 antibody-HRP conjugate (Abcam) and anti-actin antibody (Wako, Osaka, Japan). Data were analysed using LAS-3000 (FUJIFILM, Tokyo, Japan).

RNA isolation from cholangiocarcinoma cell lines and qRT-PCR

Total RNA was extracted using TRI REAGENT (Molecular Research Center, Cincinnati, OH, USA) and Direct-zol RNA mini-prep plus kit (Zymo Research, Orange, CA, USA). MiR-31-5p expression was analysed by qRT-PCR using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and TaqMan microRNA Assay (Applied Biosystems). U6 snRNA (RNU6B; Applied Biosystems) was used as an endogenous control. For the analysis of suppressor of cytokine signalling 3 (SOCS3) expression, complementary DNA was synthesised using the Super script VILO cDNA Synthesis Kit and Master Mix (Thermo Fisher, Waltham, MA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was used to normalise variance. qRT-PCR was performed using the PowerUp SYBR green master mix (Thermo Fisher) and 7500 Real Time PCR system (Applied Biosystems). All the genes were assessed in triplicates. The following primer sequences were used: GAPDH, forward primer, 5′-CAACAGCCTCAAGATCATCAG-3′, reverse primer, 5′-CTGTGGTCATGAGTCCTTCC-3′; IL-6, forward primer, 5′-CAGTTCCTGCAGAAAAAGGCAA-3′, reverse primer, 5′-AGCTGCGCAGAATGAGATGA-3′; IL-6R, forward primer, 5′-CCCATGCAGGCACTTACTAC-3′, reverse primer, 5′-TCCAGCAACCAGGAATGTGG-3′; SOCS3, forward primer, 5′-CGGAGACTTCGATTCGGGAC-3′, reverse primer, 5′-GCTGGTACTCGCTCTTGGAG-3′.

Assays for proliferation

The proliferation of cholangiocarcinoma cells stimulated with IL-6 was analysed by measuring the uptake of WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] in a colorimetric assay (CCK-8 assay, Dojindo, Tokyo, Japan).

Briefly, cholangiocarcinoma cells showing 70% confluency were dispensed into 96-well plates at a density of 3 × 103 cells per well with RPMI-1640 containing 2% FBS and 0 ng mL^{-1}, 20 ng mL^{-1} and 50 ng mL^{-1} of IL-6. After incubation for 0, 48 and 96 h, 10 μL of CCK-8 or medium was added to each well, and the plate was then incubated for 2 h at 37°C, and absorbance was measured at 450 nm on Multiskan FC (Thermo Fisher) using a CCK-8 kit as per manufacturer's instructions.

Statistical analysis

JMP (version 10) software programs were used for statistical analyses (SAS Institute, Cary, NC, USA). All *P*-values were two-sided. Univariate analyses were performed to investigate clinical and pathological characteristics according to miR-31 expression. Chi-square or Fisher's exact test was used for categorical data, while analysis of variance was used to compare the mean patient age and tumour size.

Kaplan–Meier method and log-rank test were used to assess the association between miR-31 expression status and patient survival. Additionally, Cox proportional hazards regression models were used to compute the mortality hazard ratio (HR) according to miR-31 expression status. The multivariate Cox model included miR-31 expression stratified by tumour size and pathological stage.

Results

MicoroRNA-31 expression in cholangiocarcinoma tissue

We collected and extracted RNA from cholangiocarcinoma cells and adjacent normal bile duct epithelial cells from7 FFPE samples using the MMI Cellcut plus microdissection system (MMI, Eching, Germany). We calculated miR-31 expression using the equation $2^{-\Delta Ct}$, where ΔCt = (Ct miR-31 − Ct U6). MiR-31 expression in cholangiocarcinoma cells was significantly higher than in normal bile duct epithelial cells (paired *t*-test; $P = 0.038$; **Figure 1a**).

Distribution of miR-31 expression in the 81 cholangiocarcinoma samples were as follows: mean, 2.36; median, 0.13; standard deviation (SD), 8.11; range, 0.00018–64.20. Samples with high miR-31 expression were defined as second-level samples in a dichotomous category for further analysis.

Association between miR-31 expression and clinical or pathological characteristics in cholangiocarcinoma

Table 1 summarises the clinical features of all 81 patients with cholangiocarcinoma according to miR-31 expression. We observed a significant association between miR-31 expression and tumour size. However, there were no significant associations between miR-31 expression and other clinical or pathological characteristics, including sex, age, tumour location, N factor, M factor and disease stage, except for tumour size (**Table 1**). We categorised pathological stages into stages I, II, III and IV for further analysis because of the difference in TNM classification according to tumour location.

MiR-31 expression and patient survival

We assessed the influence of miR-31 expression on clinical outcomes in 81 patients with cholangiocarcinoma (stages I–IV). The median follow-up time for overall survival (OS) was 25.1 months. Kaplan–Meier analysis revealed that survival was significantly shorter in the high miR-31 expression group than in the low miR-31 expression group (log-rank $P = 0.0082$; **Figure 1b**).

Univariate Cox regression analysis for overall survival revealed that significantly shorter survival in the high miR-31 expression group than in the low miR-31 expression group (HR: 2.08; 95% confidence interval [CI]: 1.22–4.19; $P = 0.0089$). Multivariate Cox regression analysis adjusted for pathological stage and tumour size revealed that survival was significantly shorter in the high miR-31 expression group than in the low miR-31 expression group (HR: 2.08; 95% CI: 1.02–4.28; $P = 0.043$).

Induction of miR-31 in cholangiocarcinoma

First, we examined miR-31 expression in four cholangiocarcinoma cell lines (HuCCT-1, HuH-28, G415 and TFK-1) and decided to use two cholangiocarcinoma cell lines (HuCCT-1, relatively high miR-31 expression cell line; TFK-1, relatively low miR-31 expression cell line [**Supplementary Figure 1a**]). To identify key cytokines for the induction of miR-31, we stimulated the cholangiocarcinoma cell lines with IL-1β, IL-6, IL-10, IL-17A and TNF-α. We confirmed that the receptor of these cytokines were expressed in the cholangiocarcinoma cell lines (data not shown). Among the cytokines, only IL-6 significantly enhanced miR-31 expression in both cholangiocarcinoma cell lines, whereas IL-10, TNF-α, IL-1β and IL-17A did not enhance miR-31 expression (**Figure 2a–e**). We also examined the expression of SOCS3 00to confirm whether miR-31 expression was regulated through the IL-6-STAT-3 signalling pathway. SOCS3 expression was significantly upregulated after IL-6 treatment of the cell lines (**Figure 2f**). Next, we examined miR-31 expression after treatment with S3I-201, which inhibits STAT-3

phosphorylation, activation, dimerisation and DNA-binding. Western blotting showed that S3I-201 significantly inhibited STAT-3 phosphorylation (**Supplementary Figure 2**). Quantitative PCR revealed that STAT-3 inhibition significantly suppressed miR-31 expression in a dose-dependent manner (**Figure 3a, b**). These results suggested that the IL-6-STAT-3 pathway induced miR-31 expression.

Functional analysis of IL-6 addition to cholangiocarcinoma cell lines

In the proliferation assay, IL-6 treatment significantly increased cell proliferation of cholangiocarcinoma cell lines compared with control (**Figure 4a, b**). S3I-201 treatment significantly inhibited the proliferation of each cell line treated with of IL-6 (**Figure 4c, d**). Taken together, these data indicated that the IL-6-STAT-3 pathway was obviously associated with tumorigenesis in cholangiocarcinoma (**Figure 5**).

Discussion

This study demonstrated that miR-31 was highly expressed in cholangiocarcinoma cells and that high miR-31 expression was significantly associated with shorter survival, independent of clinicopathological features. Additionally, IL-6 treatment in cholangiocarcinoma cell lines promoted miR-31 expression and cell proliferation in a dose-dependent manner, and the inhibition of the STAT-3 signalling pathway significantly suppressed miR-31 expression and cell proliferation. Therefore, our data suggested that miR-31, which is regulated by the IL-6-STAT-3 signalling pathway, could be a prognostic biomarker of cholangiocarcinoma.

The miR-31 gene is located at chromosome 9p21.3 and is reportedly deregulated in various human cancers.[23-25] Using microRNA array analysis, we previously reported that miR-31 expression was significantly upregulated in *BRAF*-mutated colorectal cancer when compared with wild-type colorectal cancer.[11] Moreover, we found associations between miR-31 expression and poor prognosis in colorectal cancer and pancreatic ductal adenocarcinoma (PDAC).[12, 13] Using a cholangiocarcinoma database in the present study, we revealed that high miR-31 expression was significantly associated with poor prognosis, as in colorectal cancer and PDAC. Interestingly, a positive correlation between high miR-31 expression and tumour size in cholangiocarcinomas was also observed. Previous reports demonstrated that miR-31 promoted cell proliferation in breast cancer[25] and colorectal cancer.[26] Especially in cholangiocarcinoma, miR-31 has been reported to promote cell proliferation by targeting RAS p21 GTPase Activating Protein 1 (RASA1) in vitro.[15] Consistent with the findings of previous reports, our clinical data strongly suggests that high miR-31 expression is involved in tumour growth.

Immune response is a series of events comprising recognition of pathogens or tissue damage, which involves cells and chemical mediators such as cytokines. Inflammation is a part of

the immune response to eliminate the initial cause of cell injury and initiate tissue repair.[27] However, it is widely known that chronic inflammation can increase the risk of cancer. In cholangiocarcinoma, primary sclerosing cholangitis, which causes chronic inflammation and liver injury, is an established risk factor.[2, 28] Recent studies revealed that sustained inflammation with hepatitis C virus and hepatitis B virus could result in the development of cholangiocarcinoma.[29-31] Thus, several inflammatory cytokines participate in both the initiation and progression of cancer.[32] Recently, it has been considered that several microRNAs, such as miR-31, have significant roles in immune regulation.[33, 34] In this regard, we investigated the cytokines that could affect miR-31 induction. We found that IL-6 treatment significantly increased miR-31 expression in cholangiocarcinoma cell lines.

Generally, IL-6 is known as a pro-inflammatory cytokine, and it plays an important role in the cancer microenvironment.[35] IL-6 promotes cancer cell proliferation, invasion and angiogenesis in many types of cancers.[36-42] With regard to IL-6 involvement in the differentiation of cholangiocarcinoma, it has been reported that IL-6 could induce metastasis in cholangiocarcinoma through the acquisition of epithelial-to-mesenchymal transition.[18]

In this study, we found that the cholangiocarcinoma cell lines HuCCT-1 and TFK-1 endogenously expressed IL-6 and IL-6 receptor (**Supplementary Figure 1b, c**). These findings suggested that the IL-6-STAT-3 feed forward loop might be involved in driving the tumorigenesis of cholangiocarcinomas.[35, 43] Additionally, it has been reported that not only autocrine but also extrinsic IL-6 stimulation can cause a further increase in cell proliferation. Unfortunately, our data could not prove whether IL-6 derived from cancer cells was mainly involved in cholangiocarcinoma growth when compared with that derived from immune cells. However, considering the present findings and the results of previous reports, the miR-31-IL-6 axis may play a crucial role in cholangiocarcinoma growth.

The in vitro study showed that S3I-201 treatment inhibited even innately activated IL-6-STAT-3 signalling, resulting in dramatic decrease in miR-31 expression and cell proliferation. SOCS3 is a protein that negatively regulates IL-6 receptor gp130 expression in response to IL-6 activation, which consequently prevents the continuous activation of STAT-3. To confirm whether IL-6-STAT-3 signalling certainly regulates miR-31 expression in cholangiocarcinoma, we examined SOCS3 expression. As a result, IL-6 treatment in cholangiocarcinoma cell lines significantly upregulated SOCS3 expression. Thus, our findings suggested that miR-31 is an independent prognostic factor that reflects IL-6-STAT-3 signalling in the cancer microenvironment, and miR-31 has an oncogenic role.

The present study has some limitations. Our study was limited by its cross-sectional design and bias potential (i.e. selection bias) that could have confounded the results. Nevertheless, our multivariate regression analysis was adjusted for potential clinical and pathological confounders. Although a further study is needed to confirm our results, this study is an interesting first step towards the improved understanding and diagnosis of cholangiocarcinoma.

In conclusion, high miR-31 expression was observed in cholangiocarcinoma tissue, and which was significantly associated with poor prognosis. There was a significant association between the IL-6-STAT-3 signalling pathway and both the proliferation of cholangiocarcinoma cells and the expression of miR-31. Our findings strongly suggest that miR-31 could be a promising biomarker for predicting the prognosis of patients with cholangiocarcinoma.

Acknowledgements: We thank the pathology departments of Sapporo Medical University

Hospital and Muroran City General Hospital for providing the tissue specimens.

Writing Assistance: The authors would like to thank Enago (www.enago.jp) for English language review.

Contributors:
Study concept and design: KI, KN
Data acquisition: KI, HK
Data analysis and interpretation: KI, KN
Drafting of the manuscript: KI, KN
Critical revision of the manuscript for important intellectual content: KN, HN
Statistical analysis: KI, KN
Material support: YK, IT, TH
Study supervision: KN, HN
Final approval of manuscript: all authors

Funding: This work was supported by The Japan Society for the Promotion of Science (JSPS) Grant-in-Aid for Scientific Research (grant number 16K07145 to K.N.) and JSPS Grant-in-Aid for Young Scientists (grant number 16K18423 to K.M.), Pancreas Research Foundation of Japan (to K.N.), Takeda Science Foundation (to K.M.), Daiwa Securities Health Foundation (to S.K.), and Sapporo Jikeikai Tomoiki Foundation (to S.K.),.

Competing interests: None.

Patient consent: Obtained.

Ethics approval: This study was approved by the institutional review boards of Sapporo Medical University Hospital and participating institutions.

Provenance and peer review: Not commissioned; externally peer reviewed.

References

1. Blechacz B. Cholangiocarcinoma: Current Knowledge and New Developments. Gut Liver 2017;11:13-26.

2. Razumilava N, Gores GJ. Cholangiocarcinoma. Lancet 2014;383:2168-79.

3. Rizvi S, Gores GJ. Pathogenesis, diagnosis, and management of cholangiocarcinoma. Gastroenterology 2013;145:1215-29.

4. Isomoto H. Epigenetic alterations associated with cholangiocarcinoma (review). Oncol Rep 2009;22:227-32.

5. Szafranska AE, Davison TS, John J, et al. MicroRNA expression alterations are linked to tumorigenesis and non-neoplastic processes in pancreatic ductal adenocarcinoma. Oncogene 2007;26:4442-52.

6. Meng F, Henson R, Lang M, et al. Involvement of human micro-RNA in growth and response to chemotherapy in human cholangiocarcinoma cell lines. Gastroenterology 2006;130:2113-29.

7. Guo Y, Xiong Y, Sheng Q, et al. A micro-RNA expression signature for human NAFLD progression. J Gastroenterol 2016;51:1022-30.

8. Harada K, Baba Y, Ishimoto T, et al. The role of microRNA in esophageal squamous cell carcinoma. J Gastroenterol 2016;51:520-30.

9. Romano G, Veneziano D, Acunzo M, et al. Small non-coding RNA and cancer. Carcinogenesis 2017;38:485-491.

10. Nguyen DD, Chang S. Development of Novel Therapeutic Agents by Inhibition of Oncogenic MicroRNAs. Int J Mol Sci 2017;19.

11. Nosho K, Igarashi H, Nojima M, et al. Association of microRNA-31 with BRAF mutation, colorectal cancer survival and serrated pathway. Carcinogenesis 2014;35:776-83.

12. Mitsuhashi K, Nosho K, Sukawa Y, et al. Association of Fusobacterium species in pancreatic cancer tissues with molecular features and prognosis. Oncotarget 2015;6:7209-20.

13. Kurihara H, Maruyama R, Ishiguro K, et al. The relationship between EZH2 expression and microRNA-31 in colorectal cancer and the role in evolution of the serrated pathway. Oncotarget 2016;7:12704-17.

14. Karakatsanis A, Papaconstantinou I, Gazouli M, et al. Expression of microRNAs, miR-21, miR-31, miR-122, miR-145, miR-146a, miR-200c, miR-221, miR-222, and miR-223 in patients with hepatocellular carcinoma or intrahepatic cholangiocarcinoma and its prognostic significance. Mol Carcinog 2013;52:297-303.

15. Hu C, Huang F, Deng G, et al. miR-31 promotes oncogenesis in intrahepatic cholangiocarcinoma cells via the direct suppression of RASA1. Exp Ther Med 2013;6:1265-1270.

16. Demarez C, Hubert C, Sempoux C, et al. Expression of Molecular Differentiation Markers Does Not Correlate with Histological Differentiation Grade in Intrahepatic Cholangiocarcinoma. PLoS One 2016;11:e0157140.

17. Shimura T, Shibata M, Gonda K, et al. Clinical Significance of Soluble Intercellular Adhesion Molecule-1 and Interleukin-6 in Patients with Extrahepatic Cholangiocarcinoma. J Invest Surg 2017:1-8.

18. Zhou QX, Jiang XM, Wang ZD, et al. Enhanced expression of suppresser of cytokine signaling 3 inhibits the IL-6-induced epithelial-to-mesenchymal transition and cholangiocarcinoma cell metastasis. Med Oncol 2015;32:105.

19. Johnson C, Han Y, Hughart N, et al. Interleukin-6 and its receptor, key players in hepatobiliary inflammation and cancer. Transl Gastrointest Cancer 2012;1:58-70.

20. Frampton G, Invernizzi P, Bernuzzi F, et al. Interleukin-6-driven progranulin expression increases cholangiocarcinoma growth by an Akt-dependent mechanism. Gut 2012;61:268-77.

21. Yan S, Xu Z, Lou F, et al. NF-kappaB-induced microRNA-31 promotes epidermal hyperplasia by repressing protein phosphatase 6 in psoriasis. Nat Commun 2015;6:7652.

22. Tian Y, Ma X, Lv C, et al. Stress responsive miR-31 is a major modulator of mouse intestinal stem cells during regeneration and tumorigenesis. Elife 2017;6.

23. Zhang Y, Guo J, Li D, et al. Down-regulation of miR-31 expression in gastric cancer tissues and its clinical significance. Med Oncol 2010;27:685-9.

24. Zhang T, Wang Q, Zhao D, et al. The oncogenetic role of microRNA-31 as a potential biomarker in oesophageal squamous cell carcinoma. Clin Sci (Lond) 2011;121:437-47.

25. Lv C, Li F, Li X, et al. MiR-31 promotes mammary stem cell expansion and breast tumorigenesis by suppressing Wnt signaling antagonists. Nat Commun 2017;8:1036.

26. Sun D, Yu F, Ma Y, et al. MicroRNA-31 activates the RAS pathway and functions as an oncogenic MicroRNA in human colorectal cancer by repressing RAS p21 GTPase activating protein 1 (RASA1). J Biol Chem 2013;288:9508-18.

27. Medzhitov R. Origin and physiological roles of inflammation. Nature 2008;454:428-35.

28. Chung BK, Karlsen TH, Folseraas T. Cholangiocytes in the pathogenesis of primary sclerosing cholangitis and development of cholangiocarcinoma. Biochim Biophys Acta 2017.

29. Shaib YH, El-Serag HB, Davila JA, et al. Risk factors of intrahepatic cholangiocarcinoma in the United States: a case-control study. Gastroenterology 2005;128:620-6.

30. El-Serag HB, Engels EA, Landgren O, et al. Risk of hepatobiliary and pancreatic cancers after hepatitis C virus infection: A population-based study of U.S. veterans. Hepatology 2009;49:116-23.

31. Donato F, Gelatti U, Tagger A, et al. Intrahepatic cholangiocarcinoma and hepatitis C and B virus infection, alcohol intake, and hepatolithiasis: a case-control study in Italy. Cancer Causes Control 2001;12:959-64.

32. Landskron G, De la Fuente M, Thuwajit P, et al. Chronic inflammation and cytokines in the tumor microenvironment. J Immunol Res 2014;2014:149185.

33. Zhang L, Ke F, Liu Z, et al. MicroRNA-31 negatively regulates peripherally derived regulatory T-cell generation by repressing retinoic acid-inducible protein 3. Nat Commun 2015;6:7639.

34. Moffett HF, Cartwright ANR, Kim HJ, et al. The microRNA miR-31 inhibits CD8(+) T cell function in chronic viral infection. Nat Immunol 2017;18:791-799.

35. Grivennikov S, Karin M. Autocrine IL-6 signaling: a key event in tumorigenesis? Cancer Cell 2008;13:7-9.

36. Kumari N, Dwarakanath BS, Das A, et al. Role of interleukin-6 in cancer progression and therapeutic resistance. Tumour Biol 2016;37:11553-11572.

37. Maccio A, Madeddu C. The role of interleukin-6 in the evolution of ovarian cancer: clinical and prognostic implications--a review. J Mol Med (Berl) 2013;91:1355-68.

38. Dethlefsen C, Hojfeldt G, Hojman P. The role of intratumoral and systemic IL-6 in breast cancer. Breast Cancer Res Treat 2013;138:657-64.

39. Chen MF, Chen PT, Lu MS, et al. IL-6 expression predicts treatment response and outcome in squamous cell carcinoma of the esophagus. Mol Cancer 2013;12:26.

40. Waldner MJ, Foersch S, Neurath MF. Interleukin-6--a key regulator of colorectal cancer development. Int J Biol Sci 2012;8:1248-53.

41. Culig Z, Puhr M. Interleukin-6: a multifunctional targetable cytokine in human prostate cancer. Mol Cell Endocrinol 2012;360:52-8.

42. Wei LH, Kuo ML, Chen CA, et al. Interleukin-6 promotes cervical tumor growth by VEGF-dependent angiogenesis via a STAT3 pathway. Oncogene 2003;22:1517-27.

43. Chang Q, Bournazou E, Sansone P, et al. The IL-6/JAK/Stat3 feed-forward loop drives tumorigenesis and metastasis. Neoplasia 2013;15:848-62.

Table 1. Clinicopathological features of 81 cholangiocarcinoma according to miR-31 expression.

Clinicopathological feature	Total N	miR-31 expression		*P*
		High expression	Low expression	
All cases	81	41	40	
Gender				
Male	48 (59%)	27 (66%)	21 (53%)	0.22
Female	33 (41%)	14 (34%)	19 (48%)	
Age (mean ± SD)	70.8 ± 9.1	69.0 ± 10.0	72.6 ± 7.8	0.18
Tumour size (mm) (mean ± SD)	32.7 ± 19.9	38.6 ± 21.3	26.9 ± 16.6	0.012 *
Tumour location				
Intrahepatic	16 (20%)	10 (24%)	6 (15%)	0.11
Extrahepatic	37 (46%)	14 (34%)	23 (58%)	
Gall bladder	28 (34%)	17 (41%)	11 (28%)	
N factor				
N0	53 (65%)	27 (66%)	26 (65%)	0.94
N1	28 (35%)	14 (34%)	14 (35%)	
M factor				
M0	78 (96%)	39 (95%)	39 (98%)	0.57
M1	3 (3.7%)	2 (4.9%)	1 (2.5%)	
Pathological stage				
I	16 (20%)	7 (17%)	9 (23%)	0.29
II	31 (38%)	13 (32%)	18 (45%)	
III	23 (28%)	13 (32%)	13 (25%)	
IV	11 (14%)	8 (20%)	10 (7.5%)	

Percentage (%) indicates the proportion of cases with a specific clinicopathological feature within a given category of miR-31. *P*-values were calculated by analysis of variance for age and tumour size and by a chi-square test or Fisher's exact test for all other variables.

SD, standard deviation

Figure 1

(a) Comparison of microRNA-31 (miR-31) expression between cholangiocarcinoma cells and adjacent normal bile duct epithelial cells (n = 7).
MiR-31 expression in cholangiocarcinoma cells is significantly higher than in normal bile duct epithelial cells (paired *t*-test; *P* = 0.038).

(b) Kaplan–Meier curves of overall survival in patients with cholangiocarcinoma according to miR-31 expression. In the Kaplan–Meier analysis, survival is significantly shorter in the high miR-31 expression group than in the low miR-31 expression group (log-rank *P* = 0.0082).

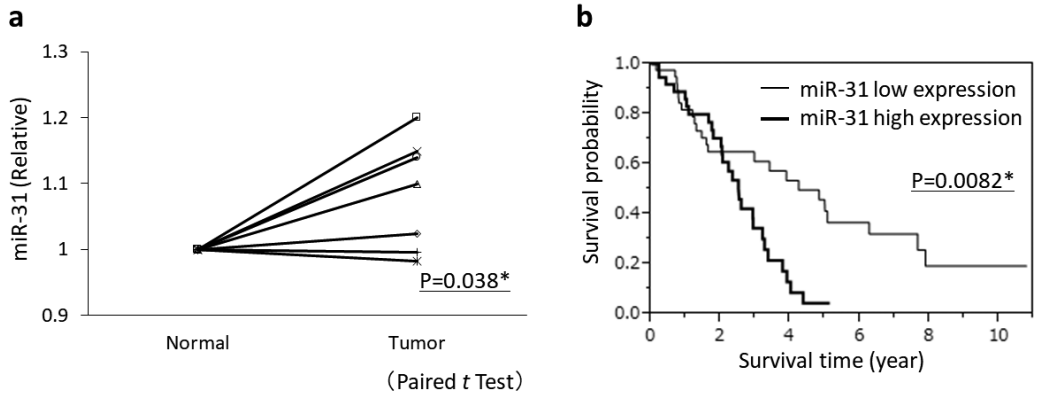


Figure 2

(a–e) Relative miR-31 expression in HuCCT-1 and TFK-1 cell lines stimulated with 0 ng·mL^{-1}, 20 ngmL^{-1} or 50 ngmL^{-1} of IL-6 **(a)**, TNF-α **(b)**, IL-10 **(c)**, IL-1β **(d)** and IL-17A **(e)**. IL-6 significantly enhances miR-31 expression in both cholangiocarcinoma cell lines.

(f) Relative expression of suppressor of cytokine signalling 3 (SOCS3) in HuCCT-1 and TFK-1 cell lines. SOCS3 expression is significantly upregulated after IL-6 treatment. $^{**}P < 0.005$

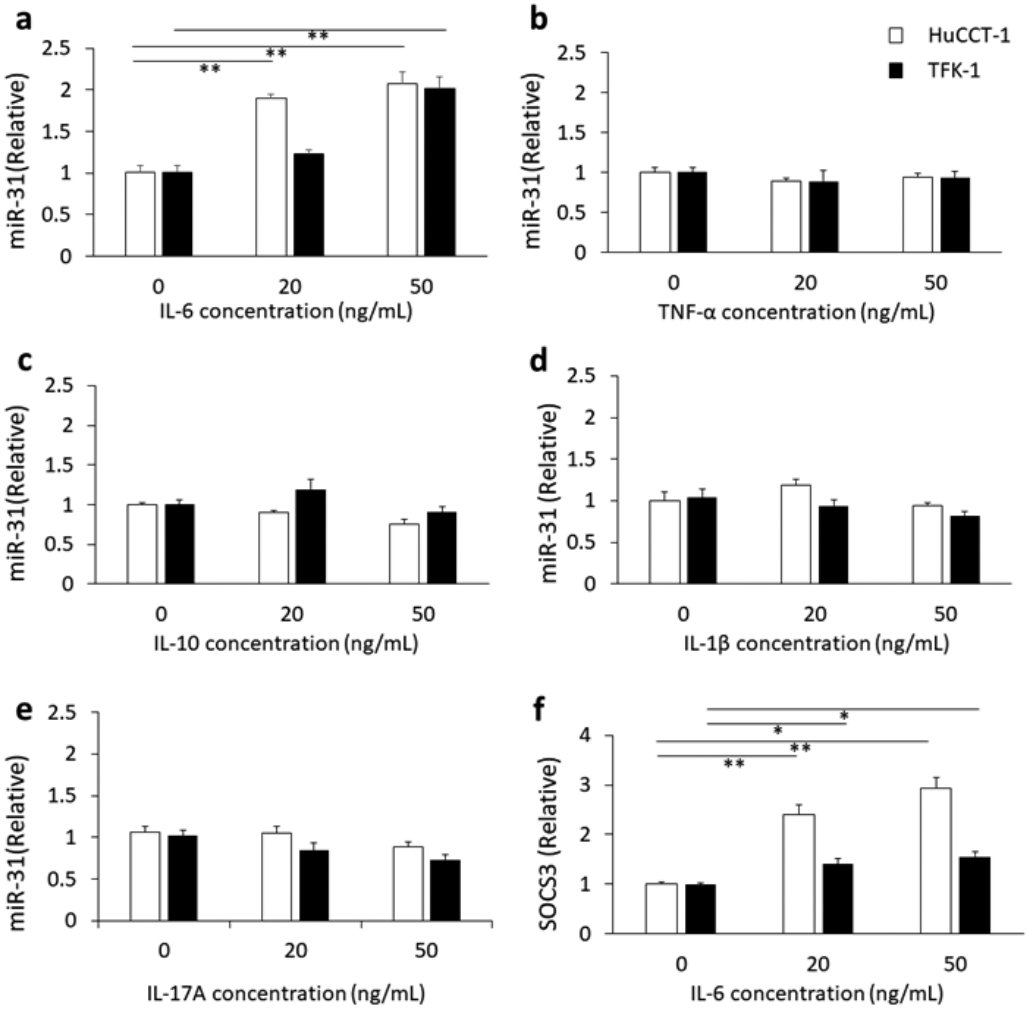


Figure 3

Relative miR-31 expression in HuCCT-1 **(a)** and TFK-1 **(b)** cell lines stimulated with 0 $ngmL^{-1}$, 20 $ngmL^{-1}$ or 50 $ngmL^{-1}$ of IL-6, and 0 μM (DMSO only), 30 μM and 300 μM of S3I-201.
**P* < 0.05, ***P* < 0.005

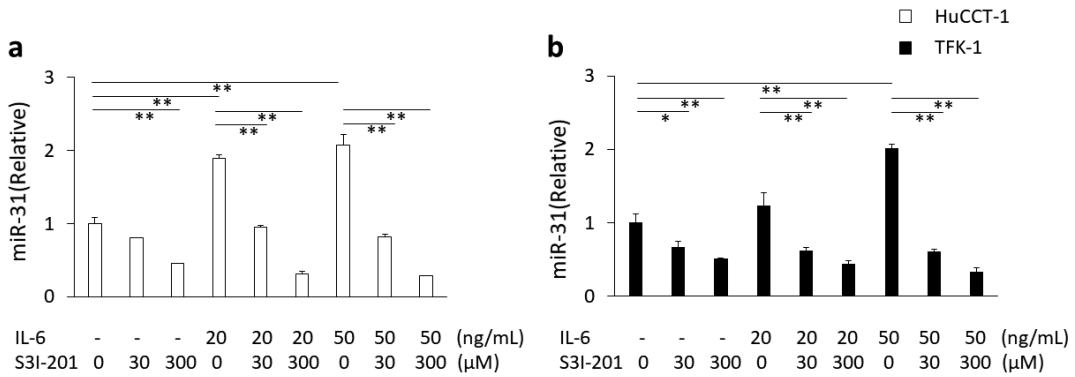


Figure 4

Results of the proliferation assay in HuCCT-1 and TFK-1 cell lines stimulated with 0 $ngmL^{-1}$, 20 $ngmL^{-1}$ and 50 $ngmL^{-1}$ of IL-6 **(a, b)**, and 0 μM (DMSO only), 30 μM and 300 μM of S3I-201 **(c, d)**. IL-6 treatment significantly enhances cell proliferation, and S3I-201 treatment significantly inhibits cell proliferation.

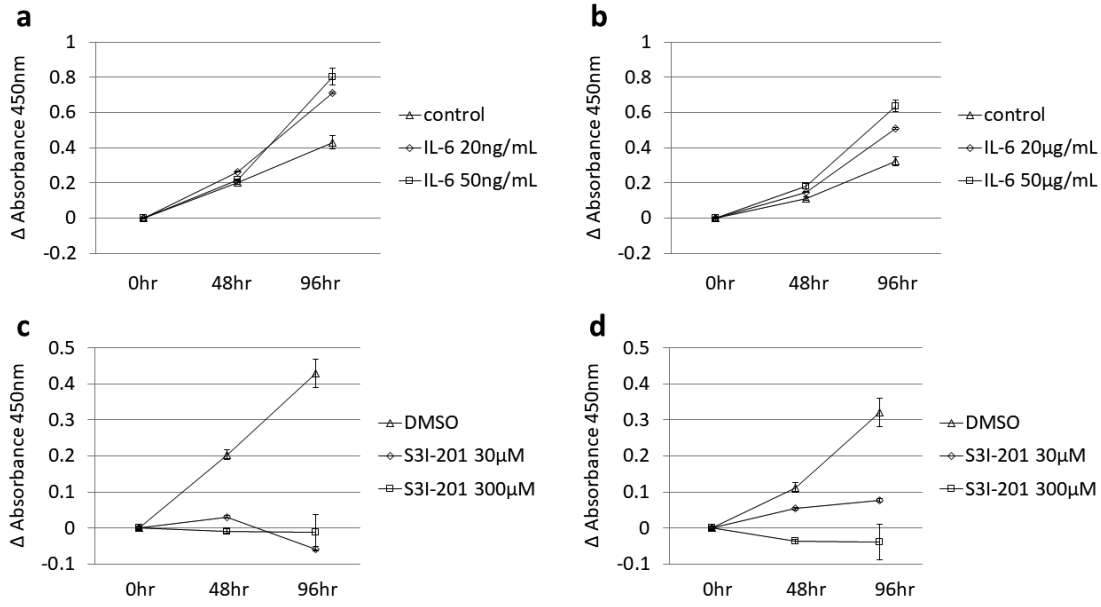
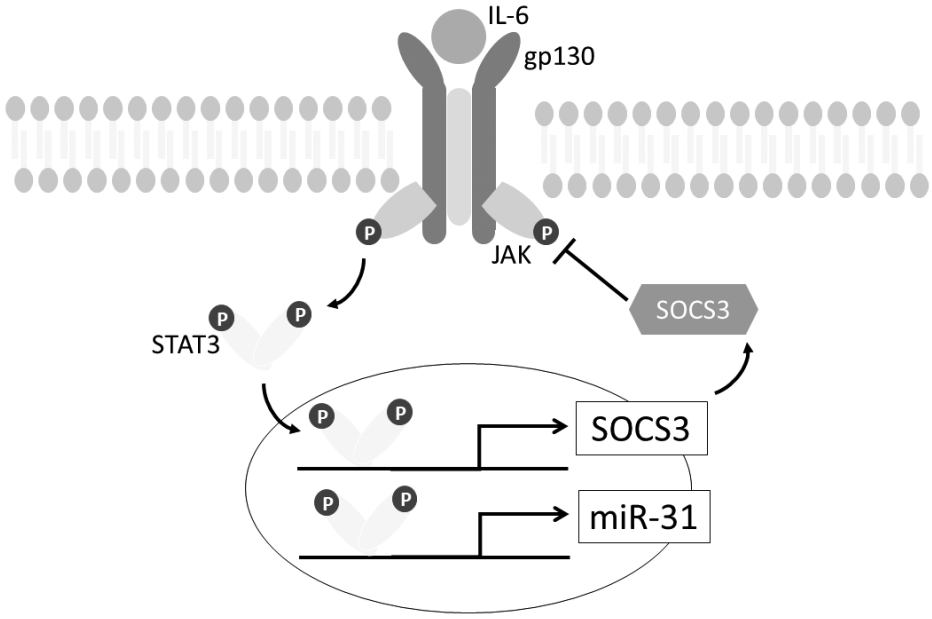


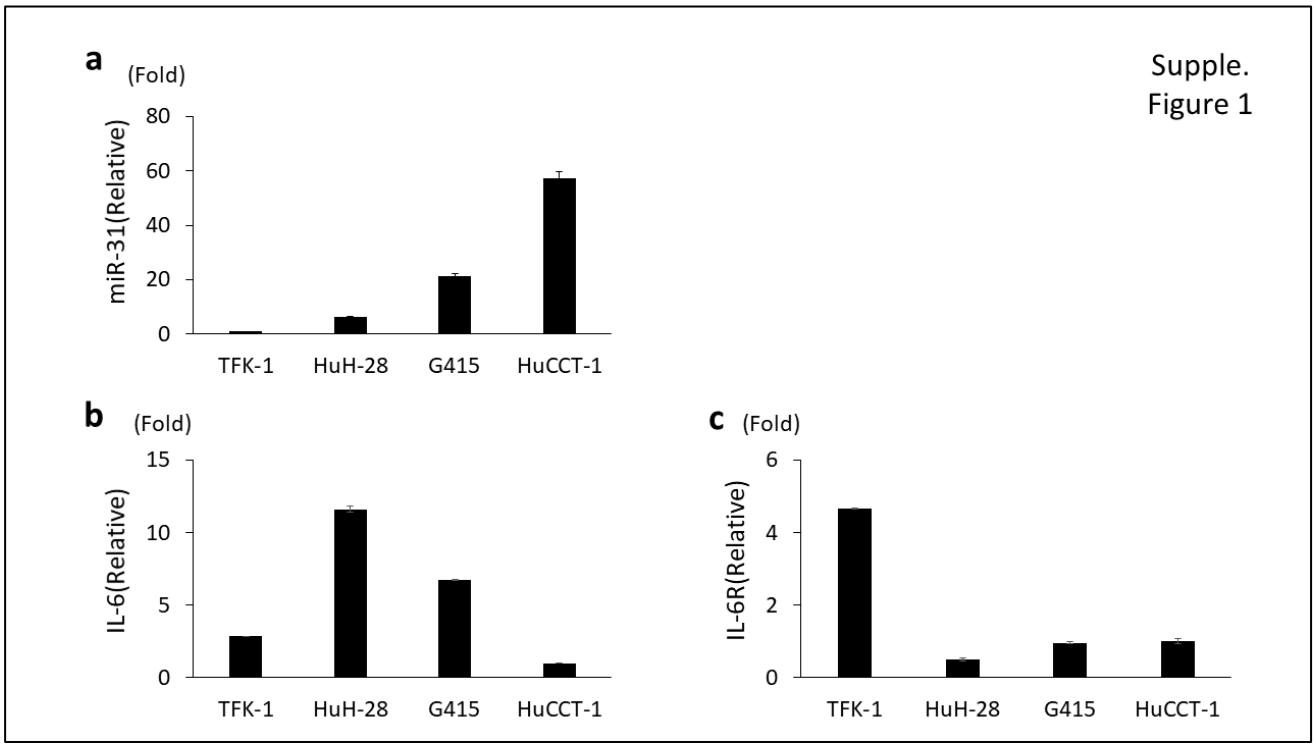
Figure 5

Scheme of the IL-6-STAT-3 pathway and miR-31
The IL-6 signal enhances miR-31 expression.



Supplementary Figure 1

Relative expressions of miR-31 **(a)**, IL-6 **(b)** and IL-6 receptor **(c)** in cholangiocarcinoma cell lines



Supplementary Figure 2

Relative phosphorylated STAT-3, total STAT-3 and actin β expressions after treatment with S3I-201
One representative experiment of the three is shown.