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Identification of transforming activity of free fatty acid receptor 2 by retroviral expression screening

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Gallbladder cancer (GBC) is a highly fatal malignancy in humans. Genetic alterations in *KRAS* or *TP53* as well as overexpression of *ERBB2* have been shown to contribute to the development of certain types of GBC. However, many cases of GBC do not harbor such genetic changes, with other transforming events awaiting discovery. We here tried to identify novel cancer-promoting genes in GBC, with the use of a retroviral cDNA expression library. A retroviral cDNA expression library was constructed from a surgically resected clinical specimen of GBC, and was used to infect 3T3 fibroblasts in a focus formation assay. cDNA incorporated into the transformed foci was rescued by PCR. One such cDNA was found to encode free fatty acid receptor 2 (FFAR2), a G protein-coupled receptor for short-chain fatty acids. The oncogenic potential of *FFAR2* was confirmed both *in vitro* with the focus formation assay and by evaluation of cell growth in soft agar as well as *in vivo* with a tumorigenicity assay in nude mice. The isolated *FFAR2* cDNA had no sequence alterations, suggesting that upregulation of *FFAR2* expression may contribute to malignant transformation. Indeed, all of quantitative RT-PCR, *in situ* hybridization, and immunohistochemical analyses showed that the amount of *FFAR2* mRNA and its protein product was increased in digestive tract cancer specimens. Furthermore, short-chain fatty acids potentiated the mitogenic action of FFAR2 in 3T3 cells. Our data thus, for the first time, implicate FFAR2 in carcinogenesis of the digestive tract. (*Cancer Sci* 2010; 101: 54–59)

Gallbladder cancer (GBC) is a highly fatal malignancy in humans, being most prevalent in South America and Asia. In most cases, GBC is not diagnosed until it has reached an advanced stage, when the 5-year survival rate is ~10%.[1,2] In the USA, ~8000 new cases of biliary tract cancer (BTC) are diagnosed each year, with ~4000 of the affected individuals subsequently dying of GBC.[3] Several risk factors have been identified for GBC, including cholelithiasis[4] and anomalous pancreaticobiliary duct junction.[5] Genetic alterations in *KRAS* or *TP53* as well as overexpression of *ERBB2* have been shown to contribute to the development of certain types of GBC. However, many cases of GBC do not harbor such genetic changes, with other transforming events awaiting discovery.

The focus formation assay with 3T3 or RAT1 fibroblasts has been used extensively to screen for transforming genes in various carcinomas.[6] In such screening, genomic DNA is isolated from cancer specimens and used to transfect fibroblasts, potentially resulting in the development of transformed cell foci. However, given that expression of the introduced genes is controlled by their own promoters or enhancers, oncogenes in cancer cells may exert effects in fibroblasts only when their control regions are active in these cells, which is not guaranteed.

Adequate expression of cDNA in fibroblasts can be achieved by placing them under the control of an exogenous promoter fragment. Toward this goal, we have recently established a retroviral cDNA expression library system that is sensitive enough to generate libraries with a high complexity even from small amounts of materials such as clinical specimens.[7–9] With this system, we have successfully discovered a fusion-type protein tyrosine kinase EML4–ALK in non-small cell lung cancer.[7]

In this manuscript, we have applied this technology to a surgically resected clinical specimen of GBC, and used this library to screen for transforming genes in GBC. Unexpectedly, transforming ability has been discovered for free fatty acid receptor 2 (FFAR2, also known as GPR43), which functions as a cellular receptor for short-chain fatty acids (SCFA).[10] Further, tumor-specific expression of FFAR2 has been proven among a panel of clinical specimens for GBC, gastric cancer, and colorectal cancer (CRC) by *in situ* hybridization and immunohistochemical analyses, indicating tumor-promoting activity among digestive tract cancers.

Materials and Methods

Clinical specimens and cells lines. Resected clinical materials were obtained from individuals who underwent surgery at Jichi Medical University Hospital. Written informed consent was obtained from each subject according to the protocols approved by the ethics committees of Jichi Medical University. Mouse 3T3 and BOSC23 cell lines were obtained from American Type Culture Collection (Manassas, VA, USA), and maintained in Dulbecco's modified Eagle medium/F12 (DMEM/F12; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Invitrogen) and 2 mM L-glutamine.

Construction of retroviral cDNA expression library. The retroviral cDNA library was constructed as described previously.[7–9,11] Briefly, first-strand cDNA was synthesized from the RNA with the use of PowerScript reverse transcriptase, the SMART IIA oligonucleotide, and CDS primer IIA (all from Clontech, Mountain View, CA, USA). The resulting cDNA was then amplified by PCR with 5′-PCR primer IIA (Clontech) and PrimeSTAR HS DNA polymerase (Takara Bio, Otsu, Shiga, Japan) for 17 cycles of 98°C for 10 s and 68°C for 6 min. The PCR products were ligated to a BstXI adapter (Invitrogen) and then incorporated into the pMXS retroviral plasmid (kindly provided by T. Kitamura of the Institute of Medical Science, University of Tokyo).

Recombinant retroviruses were produced by introduction of the plasmid library into the packaging cell line BOSC23[12] and were used to infect 3T3 cells in the presence of polybrene (4 μg/mL; Sigma, St Louis, MO, USA). The cells were cultured for 2 weeks, after which transformed foci were isolated, expanded, and subjected to extraction of genomic DNA. Insert

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cDNA was recovered from the genomic DNA by PCR with 5′-PCR primer IIA and PrimeSTAR HS DNA polymerase. Amplified products were then ligated to the plasmid pT7Blue-2 (Novagen, Madison, WI, USA) and subjected to nucleotide sequencing.

Transformation assay. For a focus formation assay, recombinant retrovirus was used to infect 3T3 cells for 48 h. The culture medium of 3T3 cells was then changed to DMEM/F12 supplemented with 5% calf serum and 2 mM L-glutamine, and incubated for 2 weeks. To examine anchorage-independent growth in soft agar, 3T3 cells infected with retrovirus were resuspended in culture medium containing 0.4% agar (SeaPlaque GTG agarose; Cambrex, East Rutherford, NJ, USA), and seeded onto a base layer of complete medium containing 0.5% agar. Cell growth was assessed after 3 weeks of incubation.

For an *in vivo* tumorigenicity assay, 3T3 cells (2×10^6) infected with the retrovirus expressing FFAR2 were resuspended in 500 μL PBS, and injected into each shoulder of *nu/nu* BALB/c mice (6 weeks old). Tumor formation was assessed after 3 weeks.

Quantitation with real-time RT-PCR. Oligo(dT)-primed cDNA was synthesized from the clinical specimens with PowerScript reverse transcriptase, and subjected to quantitative PCR with a QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA, USA) and an amplification protocol consisting of incubations at 94°C for 15 s, 60°C for 30 s, and 72°C for 60 s. Incorporation of the SYBR Green dye into PCR products was monitored in real time with an ABI PRISM 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA), thereby allowing determination of the threshold cycle (C_T) at which exponential amplification of products begins. The C_T values for cDNA corresponding to the β-actin gene (*ACTB*) and *FFAR2* were used to calculate the abundance of the latter mRNA relative to that of the former. The oligonucleotide primers used for PCR were 5′-CCATCATGAAGTGTGACGTGG-3′ and 5′-GTCCGCCTAGAAGCATTTGCG-3′ for *ACTB* and 5′-CACTCAACGCCAGTCTGGAC-3′ and 5′-TGGCATCCCTTCTCCTTGAC-3′ for *FFAR2*.

In situ hybridization with sense or antisense riboprobes corresponding to the 3′ region (nucleotides 867–1229) of the *FFAR2* cDNA isolated in this study was conducted as described previously.[13]

Immunohistochemistry. Human tissues were fixed in 4% formaldehyde in PBS at room temperature overnight, embedded in paraffin, and sectioned at a thickness of 3 μm. Sections were mounted on glass slides, deparaffinized through three changes of xylene for 4 min each, and rehydrated in distilled water through a series of graded alcohols. For histological evaluation, sections were stained with hematoxylin–eosin solutions. For immunohistochemical experiments, antigenicity was enhanced by boiling the sections in 10 mM citrate buffer (pH 6.0) in a microwave oven for 15 min, and the endogenous peroxidase activity was blocked by incubation in methanol containing 0.3% H_2O_2 for 30 min. After two washes with PBS containing 1% Triton X-100, the sections were preincubated with the blocking buffer (#X0909; Dako, Glostrup, Denmark) in a humidified chamber for 20 min at room temperature, and then incubated with anti-FFAR2 antibody (SP4226P; Acris Antibodies, Schillerstaβe, Herford, Germany) at 4°C overnight. Next, the sections were washed in PBS and incubated with horseradish peroxidase (HRP)-labeled polymers conjugated to goat antirabbit immunoglobulin (#K4003; Dako) at 37°C for 30 min. Color development was carried out by incubating the sections with 3,3-diaminobenzidine tetrahydrochloride (Wako Pure Chemical Industries, Osaka, Japan) as a chromogenic substrate. Finally, the sections were lightly counterstained with hematoxylin, mounted, and viewed under a light microscope.

Cell proliferation assay. Mouse 3T3 cells expressing FFAR2 or not expressing FFAR2 were seeded into 96-well plates at a concentration of 4×10^3 cells/well, and incubated for 24 h with DMEM-F12 medium and 1% charcoal-treated fetal bovine serum (Invitrogen). Cells were further cultured for 48 h with 100 mM sodium acetate or 1 mM sodium butyrate, and were subjected to the cell proliferation assay with the WST-1 reagent (Clontech).

Results

Focus formation assay with a GBC library. To screen for transforming genes in digestive tract cancers, we constructed a retroviral cDNA expression library from a surgically resected GBC specimen, and obtained a total of 3.2×10^5 colony-forming units of independent plasmid clones, from which we randomly selected 20 clones and examined the incorporated cDNA. An insert of ≥500 bp was present in 16 (80%) of the plasmid clones, and the average size of these inserts was 1.48 kbp (data not shown). Infection of mouse NIH 3T3 fibroblasts with the recombinant retroviral library generated a total of 89 transformed foci (Fig. 1a). No foci were obtained for cells infected with the empty virus, whereas numerous foci were readily apparent for cells infected with a virus encoding the v-Ras oncoprotein.

Each focus obtained with the cDNA expression library was isolated, expanded independently, and used to prepare genomic DNA for recovery of retroviral inserts by PCR with the primers used originally to amplify the cDNA in construction of the library.[7] We recovered a total of 45 cDNA fragments by PCR, each of which was subjected to nucleotide sequencing in both directions. Screening of the 45 cDNA sequences against the public nucleotide sequence databases revealed that they corresponded to 19 independent genes (Supporting Information Table S1). To confirm the transforming potential of the isolated cDNA, we ligated each cDNA clone to pMXS and used the resulting retroviruses to infect 3T3 cells. The focus formation assay was carried out for cDNA corresponding to 19 independent genes, revealing reproducible transforming activity for: clone ID #2, corresponding to *ARHGEF1* (GenBank accession number NM_004706); clone ID #6, corresponding to *TBC1D3* (GenBank accession number NM_032258); clone ID #7, corresponding to *FGF4* (GenBank accession number NM_002007); and clone ID #14, corresponding to *FFAR2* (GenBank accession number NM_005306) (Fig. 1b).

FFAR2 as an oncogene. FFAR2 functions as a cellular receptor for SCFA,[10] and is expressed in the digestive tract.[14] It is thought to respond to fatty acids released in the digestive tract, but has not previously been shown to possess transforming potential. We therefore focused on FFAR2 in our subsequent analyses. Given that nucleotide sequencing of clone ID #14 did not reveal any sequence alterations compared to the published cDNA sequence of *FFAR2* (GenBank accession number NM_005306), we hypothesized that overexpression of *FFAR2* might contribute to malignant transformation.

We then assessed the transforming activity of FFAR2 in 3T3 cells with a soft-agar assay. Whereas cells infected with the empty virus did not grow in soft agar, those infected with a virus encoding v-Ras grew readily (Fig. 1b). Cells infected with a virus encoding FFAR2 also formed multiple foci in repeated experiments, indicative of the ability of FFAR2 to confer the property of anchorage-independent growth on 3T3 cells. We further tested the activity of FFAR2 in an *in vivo* tumorigenicity assay with athymic nude mice. 3T3 cells infected with the empty virus or with retroviruses encoding FFAR2 or v-Ras were thus injected subcutaneously into the mice. Tumor formation was readily apparent for the cells expressing FFAR2 or v-Ras (Fig. 1b).

Overexpression of FFAR2 in digestive tract cancers. Given that our data revealed an unexpected transforming potential of FFAR2 (at least, when it is abundantly expressed), we examined

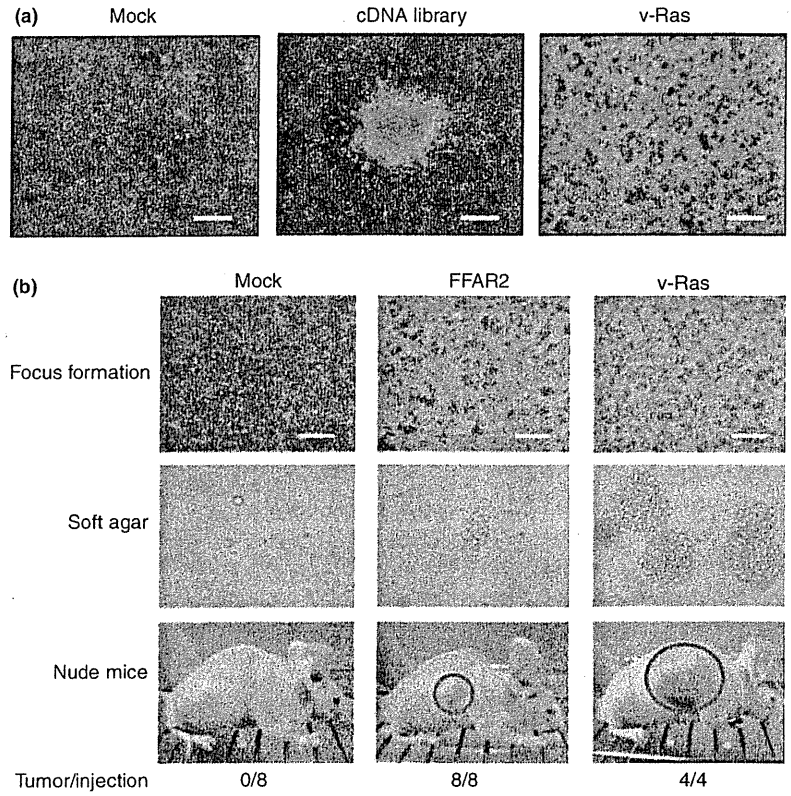

Fig. 1. Transforming activity of free fatty acid receptor 2 (FFAR2). (a) A retroviral cDNA expression library was constructed from a gallbladder cancer specimen isolated from a 64-year-old man. Mouse 3T3 cells were infected with the retroviral cDNA library, a virus encoding v-Ras, or the empty virus (Mock), and were photographed after culture for 2 weeks for the analysis of focus formation. Scale bars = 1 mm. (b) 3T3 cells were infected with viruses encoding FFAR2 or v-Ras or with the empty virus (Mock) and were then cultured for 5 days for analysis of focus formation (top panels: scale bars = 1 mm). The same batches of 3T3 cells were also assayed for anchorage-independent growth in soft agar over 17 days (middle panels) and for tumorigenicity in nude mice over 3 weeks (bottom panels). Tumors formed in the shoulders of mice injected subcutaneously with 1×10^5 cells are indicated by red circles. The frequency of tumor formation (tumor/injection) is also indicated.

whether FFAR2 might be overexpressed in human cancer specimens. We prepared oligo(dT)-primed cDNA from seven specimens of BTC, 89 specimens of gastric cancer, and 80 specimens of CRC by reverse transcription and then subjected the cDNA preparations to quantitative PCR analysis in order to measure the amount of *FFAR2* cDNA. For comparison, we also analyzed specimens of normal gallbladder ($n = 6$) and biliary duct ($n = 1$) as well as paired noncancerous tissue for all specimens of gastric cancer and CRC. Whereas the mean expression level of *FFAR2* seemed higher in BTC compared to normal gallbladder/biliary duct, a large standard deviation in the expression level made the difference insignificant ($P > 0.05$) (Fig. 2a). However, the *FFAR2* level was significantly increased ($P < 0.05$) in gastric cancer (Fig. 2b) and CRC (Fig. 2c) compared with the corresponding paired normal tissue specimens.

To examine further the site and extent of *FFAR2* expression, we carried out *in situ* hybridization analysis with a series of cancer specimens. First, a section of a CRC specimen was subjected to hybridization with sense or antisense probe for *FFAR2* mRNA. Only the antisense probe yielded clear signals in the cytoplasm and nucleus of the cancer cells (Fig. 3a), thus confirming the specificity of this probe. A series of cancer specimens was then subjected to hybridization with the antisense probe for *FFAR2* mRNA. GBC cells exhibited an increased level of hybridization compared with the normal cells in the same section (Fig. 3b). However, epithelial cells of normal gall-

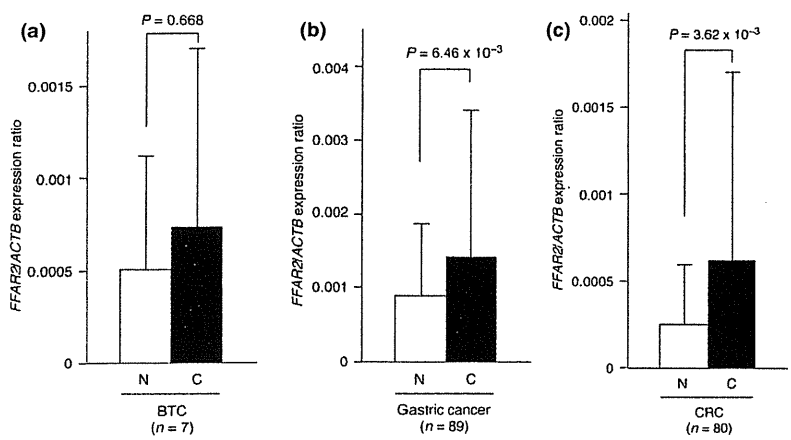

Fig. 2. Expression of free fatty acid receptor 2 (*FFAR2*) in digestive tract cancers. Oligo(dT)-primed cDNA was synthesized from (a) clinical specimens of biliary tract cancer (C) or normal gallbladder and biliary tract tissue (N), or from paired cancerous (C) and noncancerous (N) tissue specimens from patients with (b) gastric cancer or (c) colorectal cancer. The resultant cDNA was subjected to quantitative PCR analysis. Data are means + SD for the indicated *n* values, and *P*-values for the indicated comparisons were determined by Student's *t*-test. ACTB, β-actin.

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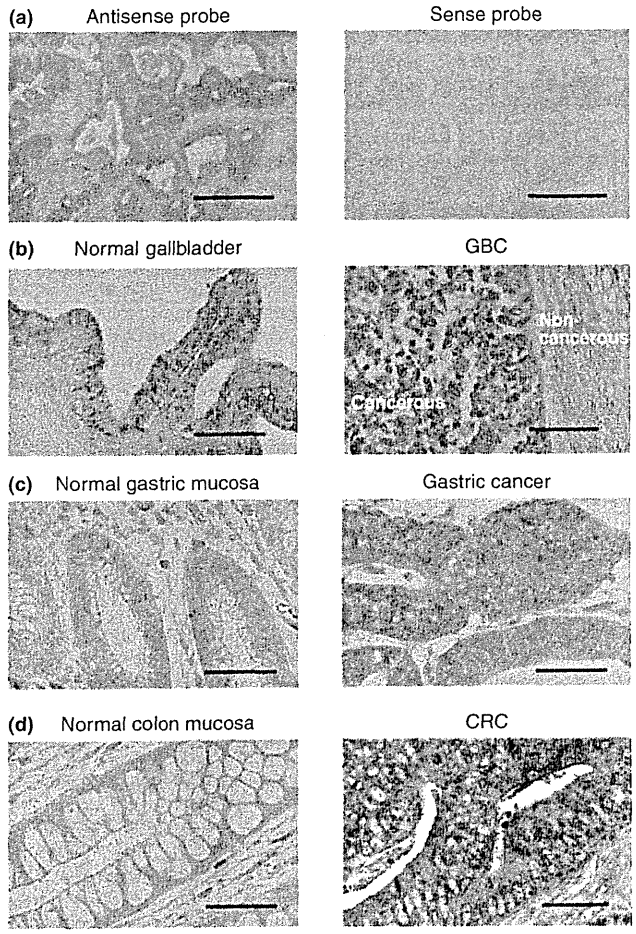

Fig. 3. *In situ* hybridization analysis of free fatty acid receptor 2 (*FFAR2*) expression. (a) A section of colorectal cancer (CRC) was subjected to *in situ* hybridization with sense or antisense riboprobes corresponding to the 3′ region (nucleotides 867–1229) of the *FFAR2* cDNA isolated in this study. (b–d) Sections of (b) normal gallbladder and gallbladder cancer (GBC), (c) paired normal gastric mucosa and gastric cancer, and (d) paired normal colon mucosa and CRC were also subjected to *in situ* hybridization with the antisense probe for *FFAR2* mRNA. Scale bars = 1 mm (a), 100 μm (b), or 50 μm (c,d).

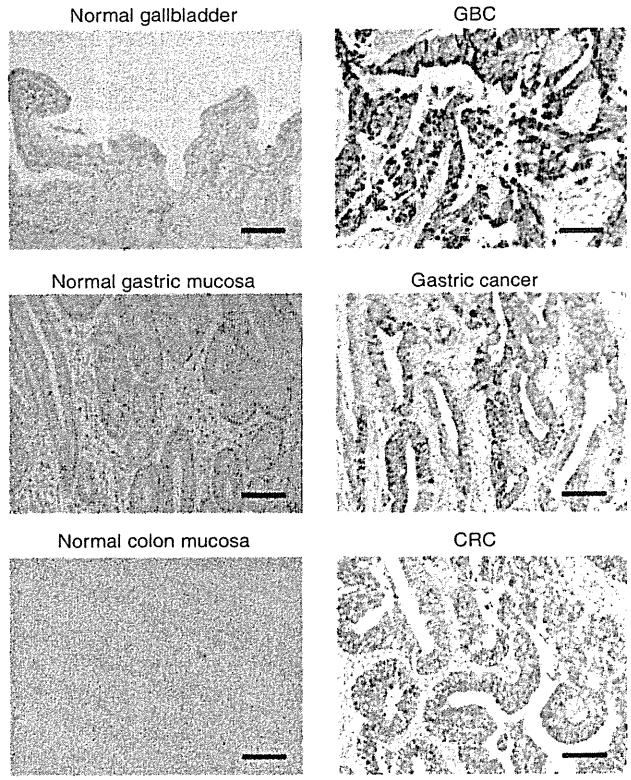

Fig. 4. Immunohistochemical analysis of free fatty acid receptor 2 (FFAR2) expression. Sections of normal gallbladder and gallbladder cancer (GBC) (upper panel), of paired normal gastric mucosa and gastric cancer (middle panel), and of paired normal colon mucosa and colorectal cancer (CRC) (lower panel) were subjected to immunohistochemical staining with antibody to FFAR2. Scale bars = 100 μm.

bladder were also stained with the probe, possibly explaining why the amount of *FFAR2* mRNA did not differ significantly between GBC and normal tissue by quantitative RT-PCR analysis (Fig. 2a). In contrast, the hybridization signal for *FFAR2* mRNA was markedly greater both in gastric cancer cells in eight of 10 specimens examined than in gland cells of the normal stomach (Fig. 3c), as well as in CRC cells in 13 of 14 specimens examined compared with the corresponding normal cells (Fig. 3d), consistent with the data obtained by quantitative RT-PCR analysis (Fig. 2b,c).

Additionally, we further examined the FFAR2 protein level by immunohistochemistry with anti-FFAR2 antibody among digestive tract cancers. As shown in Figure 4, FFAR2 was apparently induced in a GBC specimen (from which the cDNA library was generated) compared to normal gallbladder, in a gastric cancer specimen compared to its paired normal mucosa, and in a CRC specimen compared to the paired normal mucosa.

Ligand-mediated mitogenic signals of FFAR2. Given that SCFA are the presumptive ligands for FFAR2, we next examined whether the transforming activity of FFAR2 might be stimulated by its binding of such ligands. Toward this end, we incubated 3T3 cells expressing *FFAR2* cDNA in the absence or presence of the SCFA sodium acetate or sodium butyrate. Forced expression of FFAR2 induced a small increase in the growth rate of 3T3 cells even in the absence of the SCFA, whereas the SCFA had no effect on the growth of cells not expressing FFAR2. In contrast, sodium acetate (100 mM) induced a pronounced increase in the growth rate of cells expressing FFAR2 (Fig. 5a). A smaller but still significant increase in the growth rate of cells expressing FFAR2 was also induced by the addition of 1 mM sodium butyrate (Fig. 5b).

Discussion

In this study, we constructed a retroviral cDNA expression library for a GBC specimen and thereby identified the transforming potential of FFAR2. In response to its activation by ligand, FFAR2 regulates lipogenesis,[14] neutrophil migration,[15] and intestinal motility.[16] Although SCFA activate the p38 mitogen-activated protein kinase and heat shock protein 27 signaling pathway via FFAR2 in MCF-7 human breast cancer cells,[17] a relationship between FFAR2 and carcinogenesis has not previously been described.

The *FFAR2* gene has been shown to be preferentially expressed in stomach, small intestine, colon, spleen, and adipose tissue of mice.[14] A substantial amount of *FFAR2* mRNA was also detected in the rat gut, with the highest levels apparent in the colon and lower levels observed in esophagus and stom-

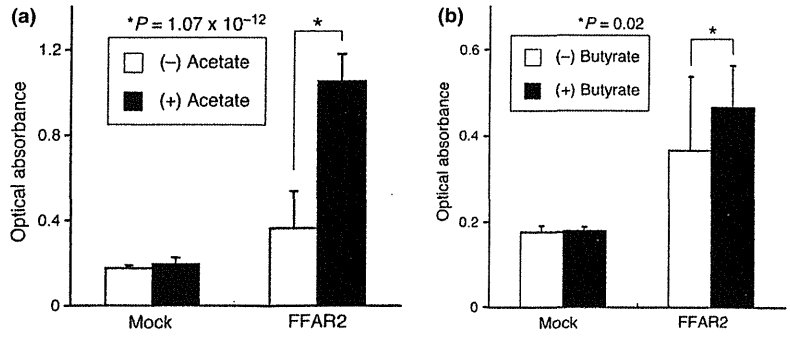

Fig. 5. Effect of short chain fatty acids on the proliferation of cells expressing free fatty acid receptor 2 (FFAR2). Mouse 3T3 cells infected with a virus encoding FFAR2 or with the empty virus (Mock) were cultured for 48 h in DMEM-F12 medium supplemented with 1% charcoal-treated fetal bovine serum in the absence or presence of (a) 100 mM sodium acetate or (b) 1 mM sodium butyrate. Cell proliferation was then assayed with the use of the WST-1 reagent. Data are expressed as absorbance at 450 nm and are means + SD of values from three independent experiments. *P*-values for the indicated comparisons were determined by Student's *t*-test.

ach.[16] In addition, FFAR2 has been detected in enterocytes of the rat intestine[18] as well as in those of the human colon.[19] The preferential expression of FFAR2 in the digestive tract and the mitogenic activity of the encoded protein together suggest a possible role for FFAR2 in carcinogenesis of the digestive system.

In our current analyses, both mRNA and protein amounts for *FFAR2* were frequently induced among the specimens for digestive tract cancer. However, DNA quantitation of the *FFAR2* locus failed to detect copy number changes of the genome (data not shown), and there are no CpG islands mapped closely or within the *FFAR2* locus in the human genome. Therefore, the molecular mechanism underlying such *FFAR2* induction is yet to be revealed.

SCFA, such as acetate, propionate, and butyrate, are the major products of the breakdown of dietary fiber by bacterial fermentation in the mammalian small and large intestine.[20] Among various SCFA, acetate has the highest selectivity for FFAR2.[21] The composition of SCFA in the colonic lumen is ~60% acetate, ~20% propionate, and ~20% butyrate.[22] SCFA are the major anions, being present at a total concentration of ~100 mM, in the lumen of the large intestine in mammals.[23] We found that the mitogenic effect of acetate in 3T3 cells expressing FFAR2 was maximal at ~100 mM (data not shown). These data suggest that FFAR2 may induce mitogenesis in the digestive tract in a manner dependent on the content of SCFA (especially that of acetate) in the diet.

It should be noted that a mere overexpression of FFAR2 significantly induced the growth of 3T3 cells even without the SCFA stimulation (Fig. 5). Although this observation potentially indicates a novel, SCFA-independent function of FFAR2, overexpression of cell surface receptors often stimulates their intracellular signaling with suboptimal concentrations of cognate ligands. Therefore, it is also possible that highly abundant FFAR2 proteins have evoked a mitogenic signaling in 3T3 in response to a low level of SCFA in the serum (or even independent of SCFA).

Diet has a substantial impact on the occurrence of digestive tract cancers, including GBC, gastric cancer, and CRC,[24] as well as on that of chronic inflammatory bowel diseases.[25] Our present findings suggest a possible connection between such disorders and either continuous exposure to SCFA in certain types of diet or induced expression of FFAR2 in the digestive tract. FFAR2 is thus a potential therapeutic target for these disorders.

Acknowledgments

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Abbreviations

ALK	anaplastic lymphoma kinase
EML4	echinoderm microtubule associated protein like-4
ERBB2	v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2
KRAS	v-ki-ras2 Kirsten rat sarcoma viral oncogene homolog
TP53	tumor protein p53

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Gallbladder cancer cDNA isolated from 3T3 transformants.

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＜症例報告＞

胆管内に進展し閉塞性黄疸をきたしたポリープ型十二指腸乳頭部癌の1例

金丸　理人[1]　小泉　大[1]　佐田　尚宏[2]

要旨：症例は61歳，男性．発熱で近医受診し，血液検査にて肝機能障害を指摘された．そのときは自然軽快したが4カ月後に再度発熱・黄疸を認め，近医で肝機能障害を指摘された．腹部エコーでは，膵頭部腫大を認め，精査目的に当院紹介となり，緊急入院となった．ENBD造影で，下部胆管に表面不整な陰影欠損を認めた．腹部造影CTでは，下部胆管内に造影効果を認める腫瘤を認めた．画像所見，臨床所見から下部胆管癌の術前診断のもと，幽門輪温存膵頭部十二指腸切除術（D2郭清）を施行した．摘出標本の病理結果は，下部胆管内に進展した十二指腸乳頭部癌（ポリープ型，adenocarcinoma，20×11mm，深達度od）であった．下部胆管内に進展したポリープ型の十二指腸乳頭部癌は極めてまれであり，文献的考察を加えて報告する．

索引用語：　十二指腸乳頭部癌　　ポリープ型　　閉塞性黄疸

はじめに

十二指腸乳頭部癌の肉眼的形態のほとんどは，腫瘤型または潰瘍型であり，ポリープ型は約1%とまれである[1]．これまでに報告されたポリープ型十二指腸乳頭部腫瘍癌は十二指腸側に進展するが，我々は胆管内に進展するポリープ型十二指腸乳頭部癌の1例を経験したので報告する．

症　例

患　者：61歳，男性

主　訴：発熱，上腹部痛，肝機能異常

既往歴：左鎖骨骨折，胃潰瘍

家族歴：特記事項なし

現病歴：2009年1月，発熱で近医受診し，肝機能障害を指摘された．そのときは自然軽快したが，5月に再度発熱・黄疸を認め，近医で肝機能障害を指摘された．腹部エコーでは，膵頭部腫大を認め，精査目的に当院紹介となり，緊急入院となった．

入院時身体所見：身長：167cm，体重：58.6kg

腹部：上腹部圧痛あり，筋性防御なし，反跳痛なし

入院時血液検査所見：血算，凝固系は正常範囲内．生化学では，T.Bil 1.01mg/dl，AST 127U/l，ALT 268U/l，ALP 1027U/l，γ-GTP 1169U/l，Amy103U/lであった．腫瘍マーカーは，CA19-9 7.9U/mlで正常範囲内であった．

腹部超音波検査：下部胆管内に14mm大の腫瘤を認める（図1a）．

内視鏡的逆行性胆管膵管造影検査（Endoscopic retrograde cholangiopancreatography）：下部胆管内に陰影欠損を認める．Endoscopic nasobiliary drainageチューブを留置した．擦過細胞診や生検は行わなかった．胆汁細胞診は生理食塩水で洗浄後，連続して3回施行しclass Ⅰであった（図1b）．ERCPの正面視画像では，腫大・発赤した乳頭部を認める（図2）．

腹部CT検査：下部胆管内に造影効果を認める腫瘤を認める（図3）．

以上から，術前診断としては下部胆管癌を疑い，手術適応と診断した．

手術所見：幽門輪温存膵頭十二指腸切除術（D2郭清，膵胃吻合）を施行した．

摘出標本所見：乳頭部～下部胆管に亜有茎性で可動性のあるポリープ型の腫瘤を認めた（図4）．

病理組織学的所見：乳頭部癌，Ab，ポリープ型，20×11mm，Papillary adenocarcinoma，深達度はod（du0，n0，panc0，bm0，em0）fStage Ⅰであった（図5）．最終診断はポリープ型の十二指腸乳頭部癌であった．

術後経過は良好で，合併症なく第22病日で退院した．本人の強い希望で術後補助化学療法（gemcitabine 1000

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受付日：平成23年3月16日
採用日：平成23年7月4日

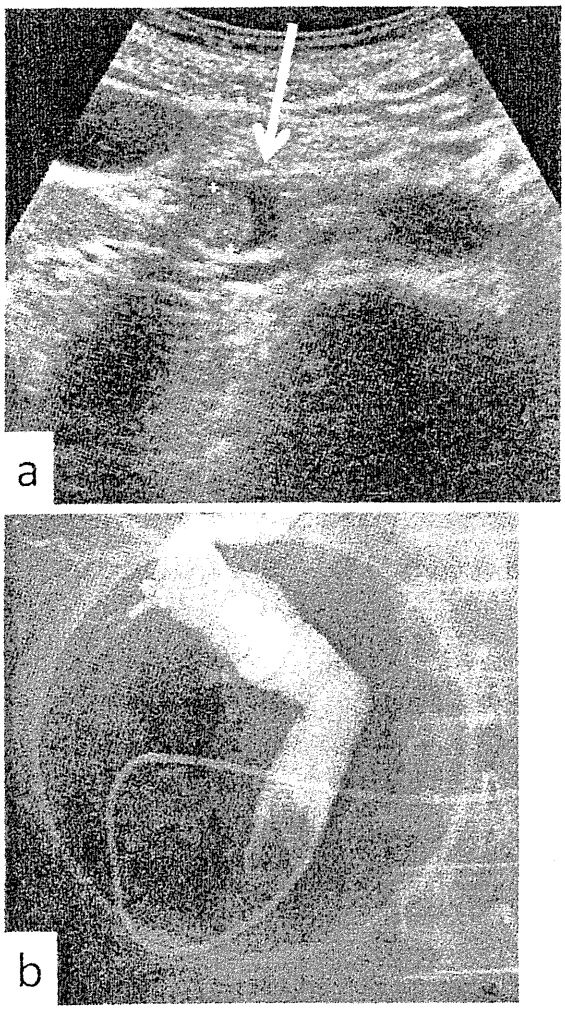


図1　a：腹部超音波検査　下部胆管内に14mmの腫瘤を認める．（矢印）
b：ENBD造影　下部胆管内に陰影欠損を認める．

mg/m², 6コース）を施行した．術後3年を経過し，無再発生存中である．

考　察

全国胆道癌登録調査報告によると1988年より2002年まで15年間に全国より登録された十二指腸乳頭部癌は1912例あるが，肉眼型が不明であった165例を除く1747例中ポリープ型はわずか20例（約1.1%）にすぎなかった[1]．

医学中央雑誌（1983年～2010年）で『乳頭部癌』，『ポリープ型』をキーワードにして検索したところ，会議録を除いた詳細な報告のあるポリープ型十二指腸乳

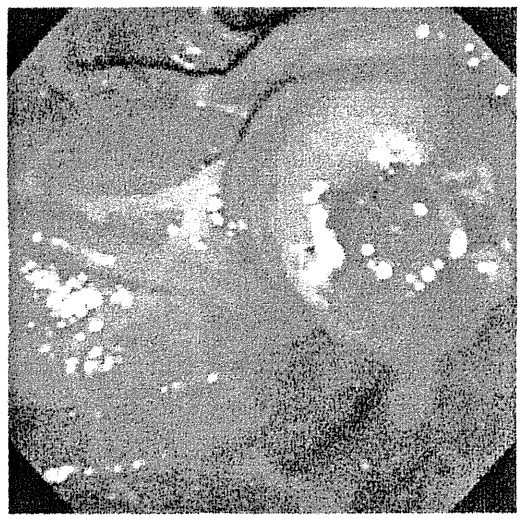


図2　ERCP正面視像
腫大・発赤した乳頭部を認める．潰瘍の形成は認めない．

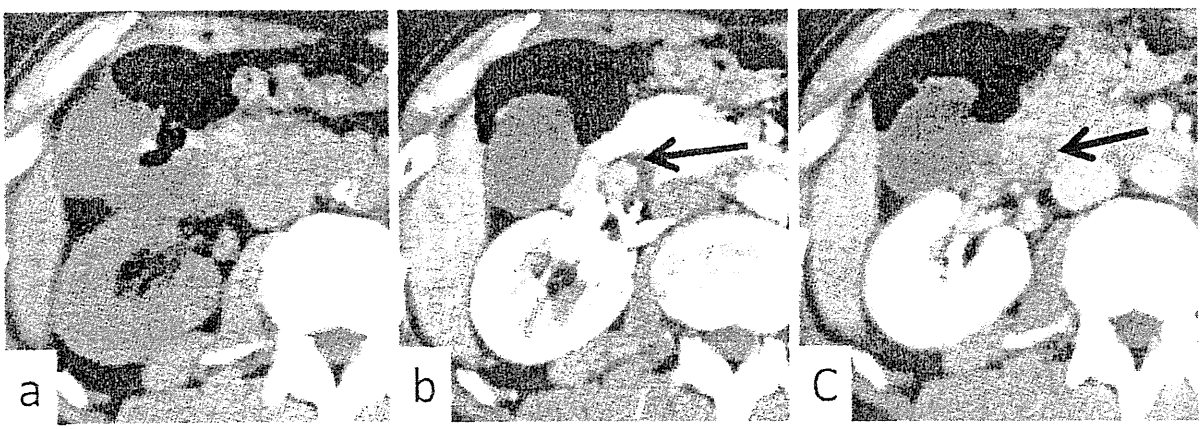


図3　腹部CT
a：単純，b：動脈相，c：門脈相
下部胆管内に動脈相にて早期濃染される14mmの腫瘤性病変を認める．（矢印）

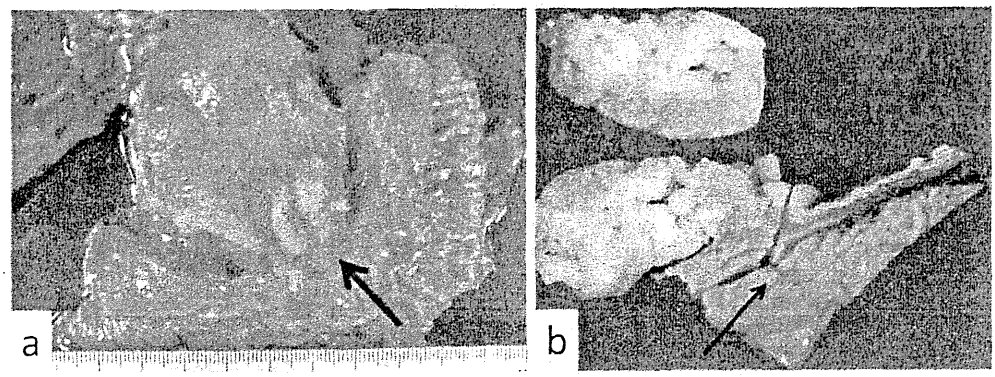


図4　摘出標本写真
a：下部胆管から乳頭部にかけてポリープ様に腫瘤を認める．（矢印）
b：aの切離面．（矢印は膵管開口部）

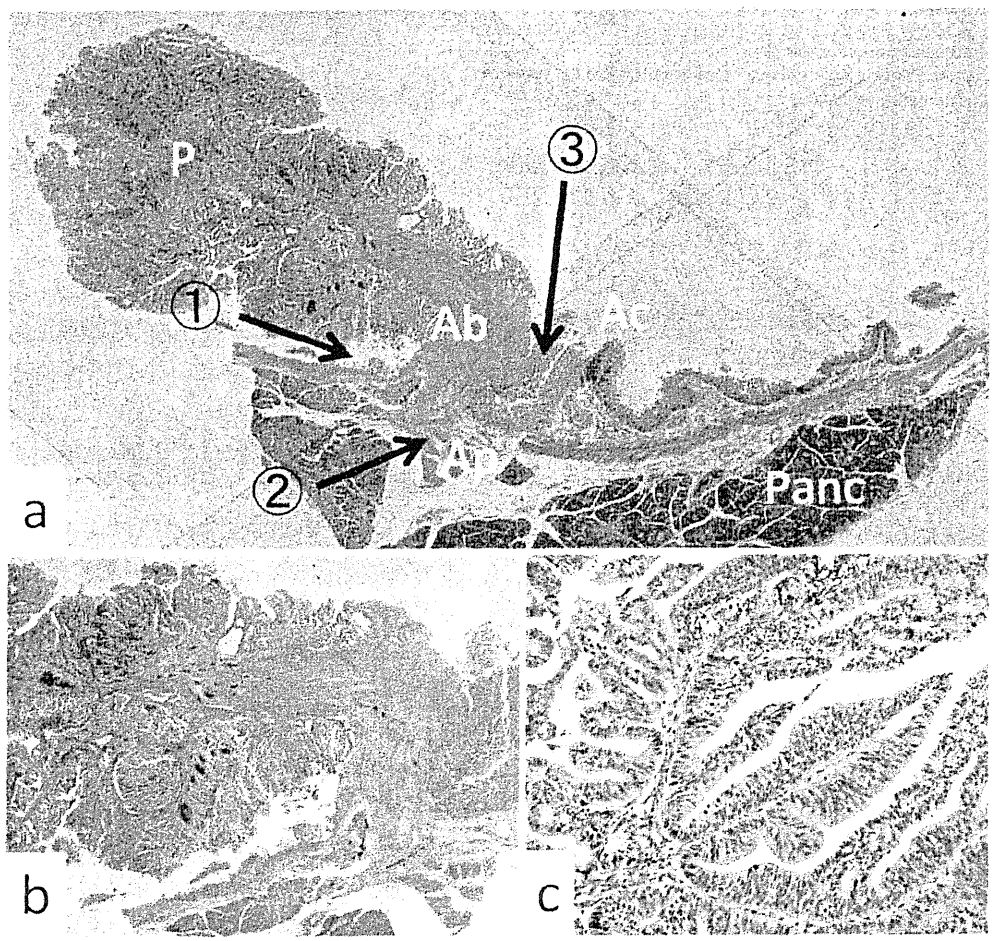
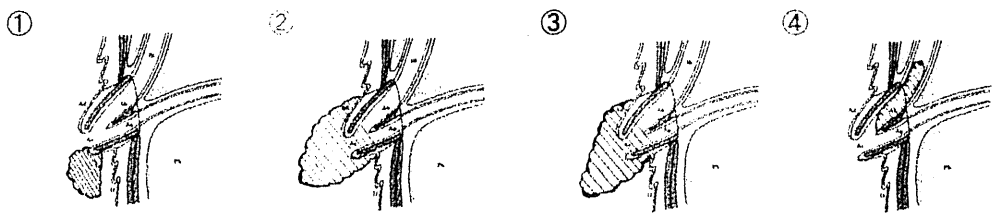


図5　病理組織学的検査
a：HE染色，ルーペ像
　1矢印：胆管，2矢印：膵管，3矢印：Oddi筋層
b：HE染色，×5
c：HE染色，×100
P：polyp，Panc：pancreas
Ab：乳頭部胆管，Ac：共通管部，Ap：乳頭部膵管

No.	報告者	報告年	年齢・性	組織型	部位	深達度	N	手術
①	神沢ら	1996	67M	pap,tub2	Ad	m	N3	PD
②	猪熊ら	2004	75F	pap	Adc	od	N0	PpPD
③	平田ら	2009	75F	pap>tub2	Acd	pDu2	N0	PD
④	自験例	2010	60歳代	pap	Ab	od	N0	PpPD



pap:papillary adenocarcinoma
tub2:moderately differentiated type tubular adenocarcinoma
PD:Pancreaticoduodenectomy
PpPD:Pylorus preserving pancreaticoduodenectomy

図6　ポリープ型十二指腸乳頭部癌本邦報告例とその発生部位（1983年～2010年，医中誌，図は胆道癌取り扱い規約第5版より改変）

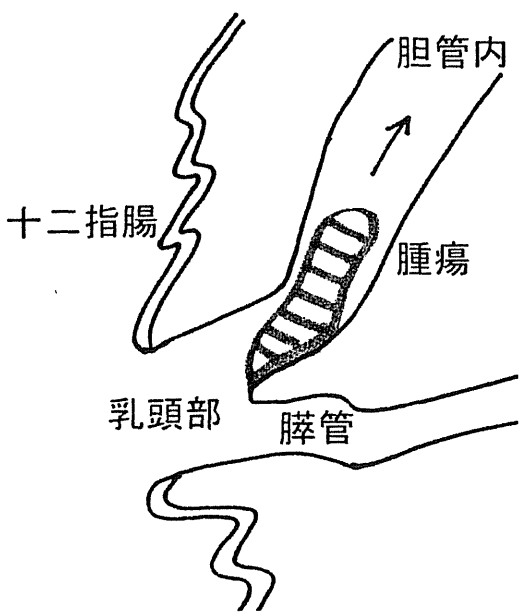


図7　本症例の腫瘍の発生部位・進展方向（シェーマ）

頭部癌は，自験例を含めて4例であった(図6)[2)～4)]．症例の平均年齢69.5歳（61～75歳），男性2例，女性2例．組織型は全例 papillary adenocarcinoma であり，2例では moderately differentiated adenocarcinoma を含んでいた．部位は Ad，Adc，Acd，Ab であった．深達度は全例 T1 であった．術式は膵頭十二指腸切除術が2例，幽門輪温存膵頭十二指腸切除術が2例行われていた．

ポリープ型の茎の発生部位を検討してみると，他の症例では Ac や Ad から発生し，腫瘍は十二指腸側に進展していたのに対して，自験例は Ab から発生し胆管内に進展しており，まれな進展形式の症例と考えられた(図6・図7)[2)～4)]．

報告のある他のポリープ型乳頭部癌3症例の発見動機は，黄疸・肝機能障害(症例①)，検診目的の腹部超音波検査（症例②），胃癌術後のフォローアップCT検査（症例③）であった．

症例①では，腫瘍はポリープ型と非露出腫瘤型の二病巣からなっており，ポリープ型腫瘍が Ad より発生し，非露出腫瘤型腫瘍が Acpd より発生していた．病理学的にはそれぞれ，乳頭腺癌，中分化型管状腺癌であった．

症例②では，腫瘍は Adc より発生していた．検診目的の腹部超音波検査で指摘され，自覚症状はなかった．ポリープ型腫瘍が十二指腸内腔側に約30mm大に発育し，黄疸は呈さず，胆管拡張が腹部超音波検査で指摘された．十二指腸乳頭部癌の発見時有症状率は早期癌でも92%であることから，この報告例のように腫瘍径

30mmで黄疸をきたさないことは通常の乳頭部癌ではまれである[2]．

症例3では，腫瘍はAcdより発生し，十二指腸浸潤を認めていた．腫瘍径が約20mmと大きいが，自覚症状はなく，肝胆道系酵素の軽度上昇と，胆管拡張を示すに過ぎなかった．本症例を含めたこれらの4症例では，いずれも発見時の自覚症状が乏しく，ポリープ型十二指腸乳頭部癌は，腫瘍の大きさに比較して症状に乏しい特徴があると考えられた．

本症例では，ERCP造影で，胆管内に凸な陰影欠損を認めたため，当初は胆管結石と考えた．しかし，造影CTにおいて同部に造影効果を認めたため，術前下部胆管に存在する腫瘍と診断した．下部胆管の「上に凸の陰影欠損」は通常結石の所見とされるが，本症例のように腫瘍である可能性も常に考えて，造影CT等での確認が必要である．

胆管癌と十二指腸乳頭部癌の鑑別診断，深達度診断において超音波内視鏡検査（EUS；Endoscopic ultrasoundscopy），膵管胆内超音波検査（IDUS；intraductal ultrasonography）などが重要であり，乳頭部癌の場合，深達度がTis（上皮内癌），T1（腫瘍進展がOddi筋にとどまる）でリンパ節転移のない早期癌であれば縮小手術（内視鏡的乳頭切除，経十二指腸的乳頭切除）が可能とする報告もあるが[3]，術前の鑑別診断，深達度診断は困難であることも少なくなく，その適応には慎重な対応が必要である．

結　語

胆管内に進展する極めてまれなポリープ型十二指腸乳頭部癌の1例を経験した．本症例と下部胆管癌との術前の鑑別は困難であるが，結石のような形態をとるポリープ型腫瘍の可能性を念頭に置くことが必要である．

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A extremely rare case of polyp type cancer of the papilla of vater which spread to lower bile duct

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A 61-years-old man went to a clinic with high fever. Laboratory data showed liver dysfunction. At the first time symptoms and data recovered naturally, but same symptoms occurred four months later and pancreatic head swelling was pointed out. For the further examination and treatment, he was admitted to our hospital. Endoscopic retrograde cholangiopancreatography revealed a mass at the low bile duct. Abdominal enhanced computed tomography scan showed the enhanced tumor. Preoperative diagnosis was lower bile duct cancer and pylorus preserving pancreatoduodenectomy was performed. Pathological diagnosis was polyp type cancer of the papilla of Vater. It was difficult to differential diagnose between lower bile duct cancer and cancer of the papilla of Vater. We herein report an extremely rare case of polyp type cancer of the papilla of Vater that spread to lower bile duct.

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Key Words: polyp type cancer, papilla of Vater, obstructive jaundice

Phase I/II Study of Hepatic Arterial Infusion Chemotherapy With Gemcitabine in Patients With Unresectable Intrahepatic Cholangiocarcinoma (JIVROSG-0301)

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Objectives: No established therapy exists for unresectable intrahepatic cholangiocarcinoma (ICC). We conducted a phase I/II study to ascertain the recommended dose (RD) of hepatic arterial infusion using gemcitabine (GEM) for ICC and to assess the efficacy and safety.

Methods: For patients with unresectable ICC, GEM was administered through the hepatic artery via the port system as a 30-minute infusion on days 1, 8, and 15 every 4 weeks for 5 cycles. In phase I, dosage for levels 1, 2, and 3 was set at 600, 800, and 1000 mg/m^2, respectively, and was increased in 3 to 6 patients at a time. Maximum tolerated dose was defined as a dosage resulting in dose-limiting toxicity in 2 of 3 patients or 3 of 6 patients, and RD was estimated during the first cycle. In the phase II, more RD patients were added to assess tumor response and toxicity.

Results: During the phase I, 16 patients were enrolled. Maximum tolerated dose was not reached. Assuming RD at 1000 mg/m^2, the phase II enrolled a total of 13 patients. The following Grade 3 toxicities were observed: neutropenia 20%, increased gamma-glutamyl transpeptidase 8%, increased aspartate aminotransferase 4%, increased alanine aminotransferase 4%, increased bilirubin 4%, nausea 4%, and fatigue 4%. The tumor response rate was 7.7% (complete response 0, partial response 1, stable disease 8, and progressive disease 4).

Conclusion: Whereas the toxicity of hepatic arterial infusion with 1000 mg/m^2 GEM for ICC was tolerable, expected efficacy could not be obtained, thus suggesting only minimal activity.

Key Words: intrahepatic cholangiocarcinoma, hepatic arterial infusion, gemcitabine, phase I/II study, clinical trial

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Intrahepatic cholangiocarcinoma (ICC) constitutes 5% to 15% of cases of the primary hepatic cancer in Japan. It is a cancer with a relatively low incidence, but is characterized by spread from the biliary epithelium to Glisson capsule. ICC has a high incidence of lymph node metastasis and vascular invasion and also tends to invade adjacent organs, so that in a fair number of cases it is already advanced and unresectable at the time of detection.[1–3] Chemotherapy is the treatment option for unresectable ICC, but no standard therapy has been established.[4,5] Typically, drug regimens centered on 5-fluorouracil (5-FU) have been used, but recently, gemcitabine hydrochloride (GEM) has appeared promising.[6]

Hepatic arterial infusion (HAI) chemotherapy is one local therapy for unresectable malignant hepatic tumors and its anticancer effect is obtained by raising the local concentration of the anticancer agent. Local therapy also reduces systemic adverse response and can increase the effect on the hepatic lesions by infusing the active medicinal agent into a hepatic artery.[7] In Japan, HAI with percutaneous placement of a catheter-port system is highly feasible,[8–10] and HAI of GEM can be continued systematically. If a local effect for ICC supplying from the hepatic artery can be obtained with HAI of GEM, this treatment may contribute to prolonging patient survival.

With this as background, we designed a phase I and II clinical trial to evaluate HAI chemotherapy with GEM for unresectable ICC, and a multicenter study was carried out by the Japan Interventional Radiology in Oncology Study Group.

MATERIALS AND METHODS

Study Design and Patient Eligibility

A phase I and II clinical trial at multiple institutions was designed to determine the dose-limiting toxicity (DLT) and recommended dose (RD) for HAI chemotherapy with GEM to treat unresectable ICC, as well as to evaluate its safety and tumor response effect. Dose-limiting toxicity and recommended dose of hepatic arterial infusion of GEM were determined as the primary end point, and the frequency and severity of adverse events, tumor response effect in the liver only, and tumor response effect in the whole body were the secondary end points. In phase I portion, DLT was assessed and RD was estimated, and in phase II portion, cases were added at the estimated RD, and the tumor response effect was evaluated. Toxicity assessment was conducted in all patients with HAI chemotherapy.

The inclusion criteria were the following conditions for cases of unresectable ICC:

1. Cases of histologically confirmed ICC (initial tumor or recurrence after resection), which was determined to be unresectable by a hepatic surgeon at each institution, or it was judged to be the prognosis-determining factor, even when metastasis was found as extrahepatic lesions.
2. Cases that were previously untreated with GEM or that were previously treated with agents other than GEM in the past, but had received no chemotherapy for at least 4 weeks from the last session, and were not responded by the chemotherapy.
3. Cases in which measurable lesions that corresponded to the target lesions on response evaluation criteria in solid tumors were located in the liver and had maximum tumor diameters of 20 mm or more

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on computed tomography (CT) images with 10-mm slices or 10 mm or more on CT images with slices of 5 mm or less.
4. Cases in which a port-catheter system for HAI was placed percutaneously, and arterially infused contrast medium was distributed through the entire liver or at least the entire hepatic lesions and in whom it was confirmed that there was no distribution of the arterially infused contrast medium in the surrounding extrahepatic organs based on CT angiography or MR angiography from the implanted port.
5. Cases aged 20 years or more with an Eastern Cooperative Oncology Group performance status classification of 2 or less.
6. Cases in which major organ function was maintained (white blood cell count $\geq$3000/mm^3 and $\leq$12,000/mm^3, platelets $\geq$100,000/mm^3, transaminase $\leq$5 times the institution's upper limit of normal, serum total bilirubin $\leq$3.0 mg/dL, serum creatinine $\leq$1.5 mg/dL, electrocardiogram not indicating the need for treatment) and in whom hepatic function was Grade 2 or less on National Cancer Institute-Common Toxicity Criteria (NCI-CTC) (version 2.0) with consideration of the influence of the hepatic lesion.
7. Cases of life expectancy of more than 8 weeks.
8. Cases in which written informed consent was obtained.

Patients excluded from the trial were the patients who scheduled for radiation therapy for the hepatic portal region because of hepatic portal region invasion or lymph node metastasis, or who had previously undergone radiation therapy; patients with concurrent infection excluding viral hepatitis, fever of 38°C or above, or who required antibiotics; patients with serious complications (intestinal paralysis, intestinal obstruction, interstitial pneumonia, pulmonary fibrosis, intractable diabetes mellitus, cardiac failure, renal failure, hepatic failure, etc); patients with other concurrent cancer; patients who could not undergo angiography because of allergy to iodinated contrast material; patients with serious mental disabilities; patients who were pregnant or may have been pregnant, and nursing mothers; and patients whose catheters for HAI chemotherapy were placed via laparotomy.

This study protocol was approved by the ethics committee of the Japanese Society of Interventional Radiology and the institutional review boards of the participating hospitals.

Treatment Protocol and Evaluation Methods

Using a percutaneously placed HAI catheter-port system, 1 course was defined as HAI of GEM on days 1, 8, and 15; a course was performed every 4 weeks for a total of 5 courses.

In phase I portion, the GEM dosage was set at Level -1, 400 mg/m^2; Level 1, 600 mg/m^2; Level 2, 800 mg/m^2; and Level 3, 1000 mg/m^2. Because the approval dosage of GEM is 1000 mg/m^2 in Japan, we defined it as the upper limit in this study. The design called for increase at each level in 3 to 6 patients from Level 1. Three patients were enrolled at each level. The study on the next dose level was not conducted until all 3 patients had completed the first cycle without any problems regarding safety and tolerance. If a DLT of any type was detected in 1 of 3 patients during the first cycle, an additional 3 patients were enrolled. If DLT was detected in more than 2 patients, the dose was defined as the maximum tolerated dose (MTD). RD was estimated to be one level below that judged to be MTD. DLT was defined as follows and judged during the first course: Grade 4 leukopenia or neutropenia; Grade 4 thrombocytopenia; nonhematologic toxicities of Grade 3 or more (excluding that from PD, nausea/vomiting, and alopecia; for patients whose pre-enrollment level of transaminase or serum total bilirubin was Grade 2, DLT was taken to be more than twice the pre-enrollment level); not meeting the criteria to start administration (same as the enrollment criteria) for the next course on day 29 because of toxicity.

In phase II portion, up to 13 patients were added at the dose found to be RD in phase I portion and the tumor response effect was judged using response evaluation criteria in solid tumors. Because HAI was being used, the target lesion was limited to hepatic lesions. Tumor size was measured on intravenous contrast-enhanced CT within 2 weeks before enrollment, and the tumor response effect was judged after the completion of courses 1, 3, and 5, and as needed.

Toxicity assessment was done in all cases using NCI-CTC (version 2.0) and the frequency of the worst grade was obtained during all courses. Physical examination and blood tests were done immediately before the start of each treatment and recorded.

Statistical Analysis

In phase I portion, the number of enrolled patients per level from Level -1 to Level 1 was minimum 6. The maximum number of patients up to Level 3, in case that MTD was reached, was 18 patients in the dose finding stage. In phase II portion, when the threshold tumor response rate was taken to be 20% and the expected efficacy rate was set at 50%, 13 patients would be needed to judge the tumor response effect under conditions of $\alpha = 0.1$ and $\beta = 0.2$, and 7 to 10 cases would need to be added at the estimated RD. For the entire study, a maximum of 25 patients was needed.

RESULTS

Patient Backgrounds

A total of 16 patients were enrolled in the phase I portion (May 2004–November 2005), and 9 patients were added for the phase II portion (February 2006–November 2006). All patients met the eligibility requirements. A summary of all 25 patients is shown in Table 1.

Phase I Portion

In phase I portion, 6 patients were registered at Level 1, 6 at Level 2, and 4 at Level 3. DLT appeared in 2 of the 6 patients at Level 1, and 2 of the 6 patients at Level 2, but DLT did not appear at Level 3. The third and fourth patients at Level 3 were registered at almost the same time. Four patients did not meet the criteria to start administration for the second course on day 29. In these 4 patients, the administration of drugs had been delayed because of Grade 1 and 2 leukopenia (n = 3) or thrombocytopenia (n = 4) in the first course. No Grade 4 hematologic toxicity or nonhematologic toxicity of Grade 3 or more was seen in the first course (Tables 2, 3). MTD was not reached up to Level 3. Accordingly, the RD was assumed to be the Level 3 dose of 1000 mg/m^2.

Phase II Portion

Nine patients were added at GEM 1000 mg/m^2. In these patients, together with the patients at Level 3 in phase I portion (total: 13 patients), the tumor response effect was complete response 0/partial response 1/stable disease 8/progressive disease 3/not evaluated 0 in the liver only, and complete response 0/partial response 1/stable disease 8/progressive disease 4/not evaluated 0 in the whole body. The response rate was 7.7% (95% confidence interval [CI], 0.2%–36.0%). Although disease control was not one of the assessment items, the disease control rate with SD added was 69% (95% CI, 38.6%–90.9%). The tumor response effect and survival in all 25 treated patients are shown in Table 4 and Figure 1.

Toxicity

The incidence of adverse events (NCI-CTC version 2.0) of Grade 3 or more in all treated cases was 20% neutropenia, 8% elevated gamma-glutamyl transpeptidase (GGT), 4% elevated aspartate aminotransferase (AST), 4% elevated alanine aminotransferase (ALT), 4% elevated bilirubin, 4% nausea, and 4% fatigue. The only

TABLE 1. Patients' Characteristics

Phase	Phase I			Phase II	
Level of GEM Dose	Level 1	Level 2	Level 3	Estimated RD	All Patients
GEM dose	600 mg/m^2	800 mg/m^2	1000 mg/m^2	1000 mg/m^2	600, 800, 1000 mg/m^2
No. patients	6	6	4	9	25
Age (yr)					
Median (range)		64 (34–76)		56 (46–74)	58 (34–76)
Gender					
Male	3	5	3	7	18
Female	3	1	1	2	7
ECOG PS					
0	4	5	3	7	19
1	1	1	1	2	5
2	1	0	0	0	1
Previous therapy					
None	4	2	3	4	13
Resection	1	3	1	5	10
Chemotherapy	1	0	1	2	4
Embolization or ablation	0	2	0	1	3
Extrahepatic lesions					
None	3	3	2	8	16
Lymph node	3	3	2	0	8
Peritoneum	1	0	0	0	1
Lung	0	1	2	1	4
Median no. courses administered	5	4.5	4		5
Median no. administrations	15	14	12		15
Relative dose intensity	81.9%	87.3%	84.8%		84.7%

ECOG indicates Eastern Cooperative Oncology Group performance status.

TABLE 2. No. Patients With Hematologic Toxicities (Cycle 1, Phase I Portion, n = 16)

	Level 1 600 mg/m^2 6				Levele 2 800 mg/m^2 6				Level 3 1000 mg/m^2 4			
Level Dose n Grade	1	2	3	4	1	2	3	4	1	2	3	4
Leucocytes	1	2	0	0	1	3	0	0	2	1	0	0
Neutrophils	0	2	1	0	1	1	2	0	1	1	0	0
Hemoglobin	0	1	0	0	0	0	0	0	0	0	0	0
Platelets	2	2	0	0	2	1	0	0	1	1	0	0

Grade 4 event was elevated bilirubin in 1 patient in the second course, but this was accompanied by portal vein tumor thrombosis (Tables 5, 6).

Events related to the HAI procedure included difficulties with the placed catheter-port system in 5 patients (catheter obstruction in 3 patients, port damage in 2 patients), and hepatic artery occlusion in 1 patient. In 2 of the patients with catheter obstruction and the 2 patients with port damage the catheter or port was exchanged and the treatment continued. The remaining patient with catheter obstruction showed an antitumor effect of PD, so the catheter was not replaced and the treatment was stopped. In the patient with hepatic artery occlusion, a left hepatic artery occlusion occurred in the second course, which meant that the drug was not reaching the left lobe of the liver, and the treatment was discontinued.

TABLE 3. No. Patients With Adverse Events (Cycle 1, Phase I Portion, n = 16)

	Level 1 600 mg/m^2 6				Level 2 800 mg/m^2 6				Level 3 1000 mg/m^2 6			
Level Dose n Grade	1	2	3	4	1	2	3	4	1	2	3	4
Nausea	0	2	0	0	2	0	0	0	3	0	0	0
Vomiting	0	1	0	0	0	0	0	0	2	0	0	0
Fatigue	1	1	0	0	3	0	0	0	0	0	0	0
Stomatitis	0	0	0	0	1	0	0	0	0	0	0	0
Headache	0	0	0	0	1	0	0	0	0	0	0	0
Diarrhea	0	0	0	0	0	0	0	0	0	0	0	0
Fever without neutropenia	0	0	0	0	0	0	0	0	1	0	0	0
Anorexia	0	0	0	0	0	0	0	0	0	0	0	0
Alopecia	0	0	0	0	1	0	0	0	0	0	0	0
Alkaline phosphatase	2	0	0	0	1	0	0	0	1	0	0	0
Bilirubin	1	0	0	0	0	0	0	0	0	0	0	0
GGT	1	0	0	0	0	1	0	0	0	0	0	0
Hypoalbuminemia	0	0	0	0	0	0	0	0	1	0	0	0
SGOT (AST)	1	0	0	0	0	0	0	0	1	0	0	0
SGPT (ALT)	0	0	0	0	0	1	0	0	1	0	0	0
Hyperkalemia	0	0	0	0	1	0	0	0	0	0	0	0
Hyponatremia	0	0	0	0	0	0	0	0	1	0	0	0

TABLE 4. Objective Response and Clinical Outcome

GEM Dose	600 mg/m²		800 mg/m²		1000 mg/m² (Phase II)		All Patients	
No. Patients	6		6		13		25	
Evaluation Site	Liver	Whole Body	Liver	Whole Body	Liver	Whole Body	Liver	Whole Body
Best response								
CR	0	0	0	0	0	0	0	0
PR	0	0	2	2	1	1	3	3
SD	4	4	3	3	9	8	16	15
PD	2	2	0	0	3	4	5	6
NE	0	0	1	1	0	0	1	1
Response rate	0%	0%	33.3%	33.3%	7.7%	7.7%	12.0%	12.0%
95% CI	0%–45.9%	0%–45.9%	4.3%–77.7%	4.3%–77.7%	0.2%–36.0%	0.2%–36.0%	2.5%–31.2%	2.5%–31.2%
Disease control rate	66.7%	66.7%	83.3%	83.3%	76.9%	69.2%	76.0%	72.0%
95% CI	22.3%–95.7%	22.3%–95.7%	35.9%–99.6%	35.9%–99.6%	46.2%–95.0%	38.6%–90.9%	54.9%–90.6%	50.6%–87.9%
Median survival time	297 d		298 d		389 d		340 d	
95% CI	140–454 d		0–747 d		158–620 d		198–482 d	

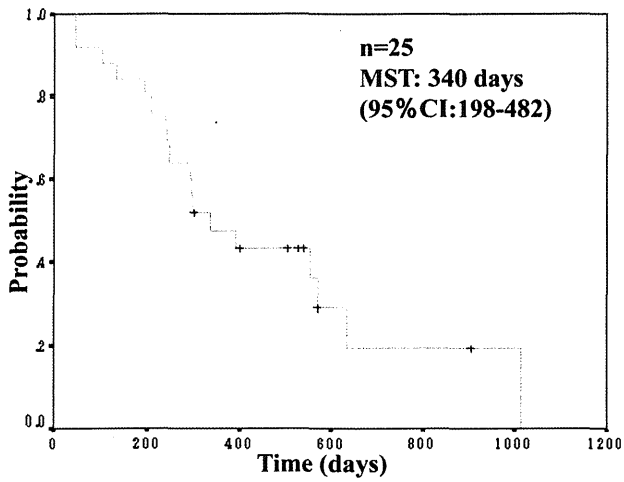

FIGURE 1. Survival time in all 25 patients received hepatic arterial infusion with gemcitabine.

TABLE 5. No. Patients With Hematologic Toxicities (Cycle 1–5, Phase I–II Portion, n = 25)

	600 mg/m²				800 mg/m²				1000 mg/m²			
Dose n	6				6				13			
Grade	1	2	3	4	1	2	3	4	1	2	3	4
Leucocytes	1	3	0	0	0	4	0	0	4	6	0	0
Neutrophils	0	2	1	0	1	1	2	0	1	7	2	0
Hemoglobin	0	1	0	0	0	1	0	0	2	1	0	0
Platelets	2	2	0	0	2	1	0	0	6	3	0	0

DISCUSSION

ICC originates in the biliary epithelium and is almost always adenocarcinoma. In Japan, it has been reported to account for 5% to 15% of primary hepatic cancers. The only curative treatment is surgical resection. However, at the time of detection, the cancer is often judged to be unresectable because of liver metastasis, vascular invasion, lymph node metastasis, or other distant metastasis.[1–3]

TABLE 6. No. Patients With Adverse Events (Cycle 1–5, Phase I–II Portion, n = 25)

	600 mg/m²				800 mg/m²				1000 mg/m²			
Dose n	6				6				13			
Grade	1	2	3	4	1	2	3	4	1	2	3	4
Nausea	0	2	0	0	3	0	1	0	7	1	0	0
Vomiting	0	0	0	0	1	1	0	0	3	0	0	0
Fatigue	1	1	0	0	3	0	1	0	3	2	0	0
Stomatitis	0	0	0	0	1	0	0	0	0	0	0	0
Headache	0	0	0	0	1	0	0	0	1	0	0	0
Diarrhea	1	0	0	0	0	0	0	0	0	0	0	0
Fever without neutropenia	0	0	0	0	1	0	0	0	4	1	0	0
Anorexia	0	0	0	0	0	0	0	0	4	1	0	0
Alopecia	0	0	0	0	1	0	0	0	1	0	0	0
Alkaline phosphatase	3	0	0	0	1	0	0	0	2	4	0	0
Bilirubin	1	0	0	0	3	0	0	0	1	1	0	1
GGT	1	0	0	0	0	1	0	0	1	0	2	0
Hypoalbuminemia	0	0	0	0	0	0	0	0	3	2	0	0
SGOT (AST)	1	0	0	0	1	0	0	0	4	2	1	0
SGPT (ALT)	0	0	0	0	1	1	0	0	3	2	1	0
Hyperkalemia	0	0	0	0	1	0	0	0	1	0	0	0
Hyponatremia	0	0	0	0	1	0	0	0	1	0	0	0

Chemotherapy is the treatment option for unresectable ICC but no standard therapy has been established.[4,5] Multiagent treatment has been reported with drugs such as 5-FU, mitomycin C (MMC), adriamycin, and epirubicin hydrochloride similar to biliary tract cancer (extrahepatic bile duct cancer, gallbladder cancer). Combined use of cisplatin and 5-FU is reportedly effective but all of these reports are from case studies only.[11,12] HAI chemotherapy has also been attempted for unresectable intrahepatic bile duct cancer and regimens such as FAM (5-FU + adriamycin + MMC), FEM (5-FU + epirubicin hydrochloride + MMC), high-dose 5-FU, and low-dose FP (5-FU + cisplatin) have been reported to be effective.[13] Again, however, all of these reports are from case studies only.

A new anticancer agent of GEM has been introduced for pancreatic cancer and biliary tract cancer, which has no standard therapy like ICC.[6] For pancreatic cancer chemotherapy, it is the drug of choice.[14,15] In treating ICC with GEM, good results were reported in 2001 from a phase II trial in Germany in which the tumor response effect was reported to be 30% and the median survival time (MST) was 9.3 months.[16] Because ICC is classified as a primary hepatic cancer in Japan, HAI of GEM has also been attempted. Tsujino et al performed HAI of GEM at the recommended dose of 1000 mg/m^2 with intravenous infusion, and they observed tumor size and tumor marker reductions.[17]

Whereas no consensus has been reached with regard to the contribution of HAI to extending survival in cases of hepatic metastasis of colorectal cancer, the local tumor response effect is considered to be superior to that with systemic chemotherapy.[18–20] Moreover, in hepatocellular carcinoma which is a primary hepatic cancer like ICC, the intra-arterial local therapy for hepatic arterial chemoembolization is thought to significantly prolong survival in unresectable cases compared with the results of symptomatic treatment.[21,22] It is possible that local therapy can also prolong survival in cases of ICC.

This study was designed with consideration of the above to establish the DLT for HAI of GEM and estimate the RD; the tumor response effect with the estimated RD was then determined and safety was evaluated. In phase I portion, GEM was increased from 600 mg/m^2 to 800 mg/m^2 and 1000 mg/m^2. A delay in the start of the second course because of Grade 1 and 2 leukopenia or thrombocytopenia as DLT was seen in 4 cases (25%). MTD was not reached up to dosage Level 3. Thus, RD was estimated to be 1000 mg/m^2, and more patients were added in phase II portion.

The incidence of adverse events of Grade 3 or more in all courses was 20% neutropenia, 8% elevated GGT, 4% elevated AST, 4% elevated ALT, 4% elevated bilirubin, 4% nausea, and 4% fatigue. The only Grade 4 event was elevated bilirubin in 1 case during the second course. However, this was a case of portal vein tumor thrombosis, which was thought to have caused the elevated bilirubin. Toxicity with HAI of GEM was generally tolerable throughout all courses and it was milder than in reports of systemic administration.[23]

Events related to the HAI itself or the implanted catheter-port system occurred in 6 cases (24%). Most were dealt with by replacing the port in order that HAI could be continued. Hepatic artery occlusion occurred in only 1 case. Compared with other reports,[8–10] more of the present cases were within the tolerable range. No catheter or port infection or induced thrombosis was observed.

The response rate of HAI of GEM at the estimated RD of 1000 mg/m^2 in 13 cases of unresectable ICC was 7.7% (CR, n = 0; PR, n = 1), which was below the established threshold efficacy rate of 20%. Although disease control was not one of the items investigated in this study, the disease control rate including SD (n = 8) was 69% and MST in all 25 patients was 340 days (95% CI: 198–482 days).

In conclusion, DLT was the delay in the start of the second course because of Grade 1 and 2 leukopenia or thrombocytopenia and RD was estimated to be 1000 mg/m^2 in HAI of GEM for unresectable ICC. Toxicity was within the tolerable range. However, the tumor response effect of HAI of GEM at 1000 mg/m^2 was low, and it was judged that no improvement in treatment results can be expected with HAI. The disease control rate and MST were acceptable, but, considering that the subjects in this study were patients whose hepatic lesions were predominant and that the implanted catheter-port system was required for HAI as a painful procedure, it cannot be claimed that this protocol has an advantage over systemic treatment.

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Outcomes and Tolerability of Systemic Chemotherapy for Pancreatic or Biliary Cancer Patients Aged 75 Years or Older

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Background: The incidence of pancreatic or biliary tract cancer is increasing in our aging population, but little is known of treatment outcomes in elderly patients with pancreatic or biliary tract cancer.
Patients and methods: Patients with pancreatic or biliary tract cancer who received chemotherapy in our institute between September 2007 and August 2009 were retrospectively reviewed to compare treatment outcomes between the elderly (aged 75 years or older) and the younger patients. Data were collected of patient backgrounds, adverse events and dose intensity within the first two cycles and overall survival time.
Results: Of the 102 who met the inclusion criteria, 19 were elderly who were introduced to full dose chemotherapy. Medication for their comorbidities was required in 15 (79%) of the 19 elderly patients and in 27 (33%) of 83 younger patients. The frequencies of haematological adverse events of grades 3 or 4 were 42% and 39%, and those of non-haematological adverse events were 21% and 16%, for the elderly and younger, respectively. Similar dose intensities were delivered to the elderly and younger. Also, similar proportions of elderly and younger received dose reductions. There was no difference in overall survival between the elderly and the younger.
Conclusion: No clear difference in treatment outcomes was seen between the elderly and the younger patients who received gemcitabine alone. Gemcitabine chemotherapy appears to be safe and the same treatment effect was seen even in older patients with pancreatic or biliary tract cancer.

Key words: elderly patients – pancreatic cancer – biliary cancer – chemotherapy

INTRODUCTION

Pancreatic or biliary tract cancer (PBCa) is known to have poor outcomes and advancing age has been associated with an increased incidence of this disease. Changing demographics in developed countries are characterized with the elderly comprising an increasing proportion of the population. This will result in a growing number of elderly patients with PBCa. In 1990, newly diagnosed pancreatic and biliary tract cancer patients aged 75 years or older showed incidences of 37% of 14 583 and 43% of 13 770 patients in Japan. However, in 2003, those numbers increased to 46% of 24 442 pancreatic cancer patients, and 58% of 11 401 biliary tract cancer patients (1). Despite the increased incidence of PBCa with age, elderly patients tend to be under-represented in clinical trials (2,3). Most clinical trials have excluded elderly patients because of the progressive reduction of organ function and co-morbidities related to age. Accordingly, only a small fraction of elderly patients have been entered into clinical trials. Hutchins reported that there was a substantial under-representation of patients 65 years of age or older in studies of treatment for cancer (4). As for lung cancer, some prospective trials showed the benefit of chemotherapy for elderly patients (5–7). However, only scant data are available among elderly patients with PBCa. Therefore, it is not known whether elderly patients

with PBCa can tolerate standard full-dose chemotherapy regimens and result in outcomes similar to those seen in younger patients. Because of this lack of evidence, chemotherapy has generally been excluded from the treatment options for elderly patients with advanced PBCa probably for the reasons that chemotherapy is never curative and has toxic effects.

To determine the safety and effectiveness of chemotherapy for elderly PBCa patients, we reviewed the data of patients who were treated at our institution.

PATIENTS AND METHODS

Patients

Patients were selected from our database with the following criteria: (i) radiologically confirmed pancreatic or biliary tract carcinoma (intrahepatic or extrahepatic cholangiocarcinoma, gallbladder carcinoma, or ampullary carcinoma), (ii) histologically or cytologically proven adenocarcinoma, (iii) no prior anti-cancer chemotherapy for PBCa, (iv) chemotherapy with gemcitabine (Gem) alone initiated between September 2007 and August 2009 at the Cancer Institute Hospital. Elderly patients were defined as 75 years of age or older, and treatment outcomes were compared between the elderly and the other patients.

Chemotherapy

In clinical practice, we generally employed Gem alone as the front line chemotherapy for PBCa. Gem was delivered at a dose of 1000 mg/m^2 by intravenous infusion on days 1, 8 and 15 of a 4-week cycle. Indicators of consensus criteria of chemotherapy in our team included good performance status, adequate organ function and historical absence of serious cardiac or cerebral vascular disease or mental disorder. For elderly patients, no geriatric assessment scoring system was used but Eastern Cooperative Oncology Group (ECOG) performance status (PS) 0 or 1 was regarded as essential for chemotherapy. However, details of indicators or treatment management depended on each physician. In general, Gem was suspended to allow recovery from the following toxicities: neutrocyte count $<1000/mm^3$, platelet count $<70\,000/mm^3$ or grade 3/4 non-haematologic toxicity.

Analysis

Individual data were collected from all medical records of the study patients. This included the past medical history, present illness, documents of imaging diagnosis, laboratory data and adverse events within the first two cycles of chemotherapy, and status at the last visit. Toxicities were graded according to the National Cancer Institute Common Toxicity Criteria version 3.0. Dose intensity was surveyed within the first two cycles for Gem monotherapy. Overall survival data was fixed on June 2010.

In the current study, the responses between the elderly (75 years or older) and the younger were subjected to statistical comparisons. This study protocol was approved by the institutional review board in Cancer Institute Hospital.

Statistical Analysis

Differences between the elderly and the younger were compared using the exact Wilcoxon test for numeric or ordinal variables, Fisher's exact test for binary variables and likelihood ratio test for multi-category discrete variables. Two-sided P values <0.05 were considered to be statistically significant. Overall survival was measured from the date of the start of chemotherapy to the date of death or last follow-up. Survival curves were generated using the Kaplan–Meier method, and median survival times were reported with 95% confidence intervals.

All statistical analyses were performed using the SPSS statistical software program package (SPSS version 11.0 for Windows).

RESULTS

Patient and Tumour Characteristics

There were 102 PBCa patients who met the selection criteria (Table 1). Of the 102, 19 (19%) were elderly patients.

The median ages were 78.0 years (range 75–85) and 65.3 years (range 41–74)) for the elderly and younger patients, respectively. The ECOG PS at the baseline was either 0 or 1 in all of the patients. The median follow-up duration was 9.1 months. There were no significant differences for background data between the elderly and the younger patients.

Comorbidities of the Patients

The comorbidities of the patients are listed in Table 2. Of the 19 elderly patients, 15 (79%) had at least one

Table 1. Patient characteristics for the elderly (aged 75 years or older) and the younger (under 75 years)

	≥75	<75	Total	*p*
No. of patients	19	83	102	
Male	12	45	77	
Female	7	38	45	0.61
Median age, years (range)	78.0 (75–85)	65.3 (41–74)		
Primary tumour site				
Pancreas	13	60	73	
Biliary	6	23	29	0.78
Status				
Locally advanced	10	35	45	
Metastatic	9	48	57	0.53

Table 2. Summary of comorbidities of the patients

Comorbidities (medication)	≥75	<75	
Cerebral disease			
Old cerebral infarction	1[a]	0	
Anticoagulant use	1		
Cardiovascular			
Hypertension	9[b]	20[c]	
Angina pectoris	1	5	
Arrhythmia	0	2	
Rheumatic aoritis	0	1	
Antihypertensive drugs	9	20	
Anticoagulant use	2	5	
Respiratory disease			
Asthma	1[d]	1	
Inhaler use	1	1	
Diabetes mellitus	6	13	
Insulin use	1	4	
Oral administration	5	9	
Renal failure	0	0	
Total cases (%)	15 (79%)	27 (33%)	$P < 0.001$

The numbers refer to patients who had one or more comorbidities.
Of 19 elderly patients, 8 patients had one, 6 had two and 1 had three kinds of comorbidities.
[a]The patient also used antihypertensive drug.
[b]Five patients also used diabetes drugs.
[c]Nine patients also used diabetes drugs.
[d]The patient also used antihypertensive drug and diabetes drugs.
Of 83 younger patients, 15 patients had one and 12 had two kinds of comorbidities.

comorbidity which needed some degree of medication. In contrast, comorbidities were seen in 27 of 83 (33%) younger patients. The proportion of the elderly with comorbidities was significantly higher than in the younger patients. Cardiovascular disease and diabetes mellitus were most frequently observed. Serum creatinine levels at the initiation of chemotherapy were under 1.5 mg/dl for all patients and severe renal dysfunction was not seen in the current study. Of the 19 elderly patients, there were 5 patients with medical history of cancer surgery: 3 with gastric cancer, 1 with breast cancer, and the remaining 1 with thyroid cancer. Of the 83 younger patients, there were 9 patients with history of cancer surgery: 1 with oesophageal cancer, 4 with gastric cancer, 3 with colorectal cancer, 2 with uterus cancer, and the remaining 1 with prostate cancer (1 with both gastric cancer and colorectal cancer).

Toxicities

Adverse events, for which grading resulted in the worst values were encountered within the first two cycles of treatment, and are reported in Table 3. Haematological toxicities of grades 3–4 were seen in 8 (42%) of the 19 elderly and in 32 (39%) of the 83 younger patients. Non-haematological toxicities occurred in 4 (21%) of the elderly, and in 13 (16%) of the younger patients. The number of any grade 3–4 toxicities were 8 (42%) in the elderly and 37 (45%) in the younger patients. Severe adverse events occurred in 1 (5%) of the elderly and in 9 (11%) of the younger patients.

Severe adverse event in the elderly patient was cholangitis due to progression of the original lesion and recovery was observed within a few days by biliary drainage. Most of the adverse events were also related to progression of PBCa.

The number of patients who could not continue chemotherapy by two cycles due to adverse events was two (11%) in the elderly and eight (10%) in the younger patients. The frequencies of any of the grade 3–4 toxicities and severe adverse events were not significantly different between the elderly and the younger patients.

Table 3. Summary of grades 3–4 toxicity in the elderly (aged 75 years or older) and the younger (under 75 years)

	No. of events		*p*
	≥75	<75	
Anaemia	0	0	
Leucopoenia	1	4	
Neutropenia	6	49	
Thrombocytopenia	2	5	
Total (*Haematological AE*) cases (%)	**8 (42%)**	**32 (39%)**	0.61
Lethargy	2	6	
Infection (non-neutropenic)	1 cholangitis	5 cholangitis	
		1 pneumoniae	
		1 liver abscess	
Infection (neutropenic)	0	2	
Bilirubin	0	2	
Transaminases	3	10	
Gastrointestinal	0	1 ascites	
1 ileus			
Diarrhoea	0	1	
Renal	0	0	
Pulmonary	0	1 interstitial pneumoniae	
Others	0	1 hypocalcaemia	
Total (*non-haematologic AE*) cases (%)	**4 (21%)**	**13 (16%)**	0.77
Total (all AE) cases (%)	**8 (42%)**	**37 (45%)**	1.00

Toxicities were evaluated according to the National Cancer Institute Common Toxicity Criteria version 3.0.