ました．これまで臨床腫瘍学をまとまって勉強する機会に恵まれなかった方，あるいは，入院の際に担当するだけでは患者さんの治療経過全体がつかみにくいと思っている方，まずは今回の特集を一読してみてください．読み終えるころには，病棟で担当するひとりひとりのがん患者さんの診療に必要な「コモン・センス」が身についているはずです．そして，がん診療の魅力を感じてさらに勉強したいと思われたら，専門病院での研修に挑戦してみてください．

文　献

1）平成21年人口動態統計．厚生労働省，2009

Profile

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40年余の歴史ある国立がん研究センターレジデント制度は現在も進化し続けています．明日のがん医療を担う若い先生方の応募をお待ちしております．研修等についてのお問い合わせは kikaku-resi@ml.res.ncc.go.jp までどうぞ．

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若い先生方が患者さんのための的確な診療や質の高い臨床研究の方法を学び，将来の日本のがん診療を担うリーダーになることをめざして，レジデント教育に取り組んでいます．日本各地から集まった仲間とともに最先端の技術や知識を学んでいるレジデントの先生たちの姿は頼もしく，私たちスタッフにもよい刺激となっています．

タルセバにおける開発の状況とその効果予測因子

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索引用語：Pancreatic Neoplasms, Epidermal growth factor receptor, Erlotinib

1 はじめに

タルセバ(erlotinib・中外製薬)は，塩酸ゲムシタビン(GEM)との併用で進行膵癌に対して，有効性が示されている．本邦では膵癌に対してはまだ保険承認されていないが，非小細胞肺癌に対してはすでに保険承認されている．

進行膵癌における治療開発において，GEMの標準治療確立後，多くの薬剤でGEMとの併用による有効性を検証する試験が実施された．しかしほとんどの試験でGEM単剤を凌駕するような成績は示されていない[1~8]．このような中，上皮増殖因子受容体(Epidermal Growth Factor Receptor: EGFR)をターゲットとしたerlotinibの有効性が報告されており，本稿ではその臨床における開発の状況と効果予測因子の可能性をまとめる．

2 EGFR

ヒト上皮増殖因子受容体(Human epidermal growth factor receptor: HER)には，HER1, HER2, HER3, HER4の4種のファミリーの存在が知られており，EGFRはHER1のことを指す．EGFRは正常組織に発現しており，これにリガンドが結合すると二量体を形成し細胞内チロシンキナーゼがリン酸化され，下流へシグナル伝達されることで細胞の分化・増殖が誘導される[9,10]．EGFRは多くの固形癌細胞でも過剰発現していることが報告されており[11~14]，癌細胞の増殖，アポトーシス抑制，浸潤，転移などに関与していると考えられている[15~19]．

3 erlotinib（タルセバ）

erlotinibはEGFRの細胞内チロシンキナーゼに選択的に結合しリン酸化を阻害する薬剤

Kohei NAKACHI et al: Clinicaltrial of gemcitabine plus erlotinib and predictive factors in pancreatic cancer
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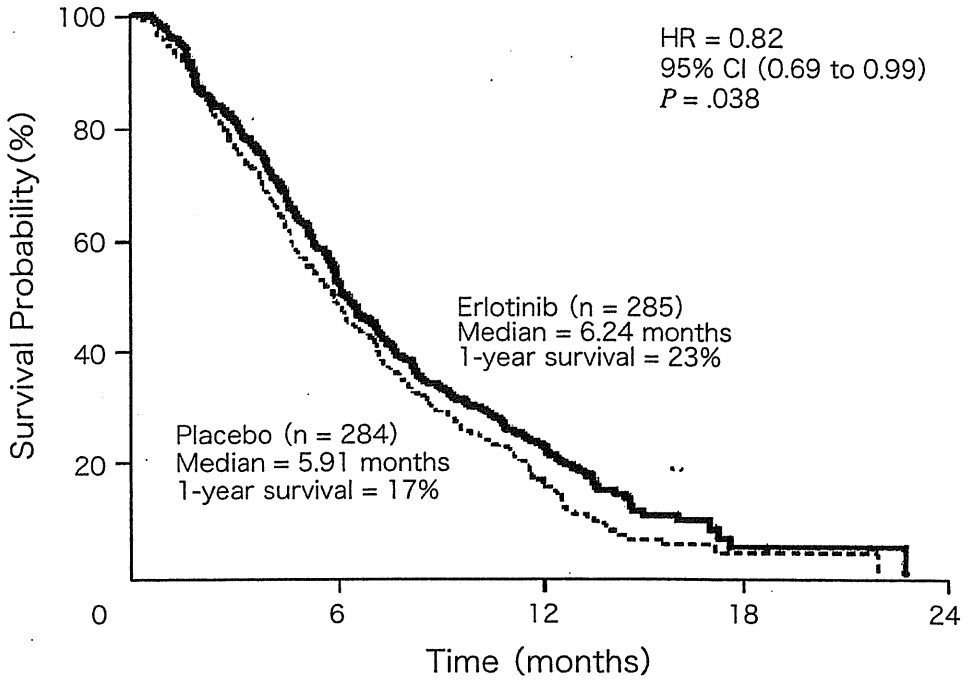

図1　PA.3試験　生存曲線

(EGFR-TKI)であり，癌細胞の増殖抑制・アポトーシス誘導・浸潤・転移抑制を促すと考えられている．膵癌においてもEGFRの過剰発現が報告されており[20,21]，erlotinibの効果が期待され臨床開発が行われた．はじめにGEM + erlotinib併用の第IB相試験として行われ，15例の進行膵癌症例に対して投与が行われた．無増悪生存期間(PFS)中央値289日，生存期間(OS)中央値389日(12.5カ月)，1年生存割合51％と良好な結果が得られた[22]．その後，カナダや米国を中心に進行膵癌に対してGEMと本剤またはplaceboとの併用の第III相試験が行われた(PA.3試験)[23]．OS(中央値)はplacebo群で5.91カ月，erlotinib群で6.24カ月と点推定値の差は僅かではあるが，ハザード比は0.82（95％ C.I. 0.69～0.99）(P = 0.038)（図1)とerlotinib群で生存期間の延長が証明され，1年生存割合もplacebo群が17％に対して，erlotinib群が23％と良好であった．主な有害事象は皮疹，下痢などであった．間質性肺疾患様事象がplacebo群で0.4％であるのに対して，erlotinib群で2.1％と高頻度に認められたが，忍容性は良好と判断されている．本試験の結果に基づき，米国，欧州では本剤が進行膵癌に対する治療薬として承認されている．

本邦でも，同併用療法の承認を目的とした単アームの第II相試験が施行された．106人の進行膵癌患者に対し同併用療法が行われ，奏効割合20.3％，OS（中央値）9.23カ月，1年生存割合33％と良好な治療成績が示された(JO20302試験）[24]．主な有害事象は皮疹や下痢であり，PA.3試験とほぼ同様なものが認められている．また間質性肺疾患の発現は9例(8.5％)に認められているが，いずれも早期に診断され，休薬やステロイド投与などにより死亡例は認められていない．PA.3試験での発現頻度と比べやや高めになっているが，その原因については明らかにはなっていない．非小細胞肺癌に対するerlotinibやgefitinibなどのEGFR-TKIによる治療においても，欧米人に比べ日本人での発現頻度が高

表　K-ras遺伝子変異と治療効果

		erlotinib群	placebo群	ハザード比	P値
生存期間(中央値)					
	K-ras変異型	6.0	7.4	1.07	0.78
	K-ras野生型	6.1	4.5	0.66	0.34
無増悪生存期間(中央値)					
	K-ras変異型	3.8	4.0	0.93	0.74
	K-ras野生型	3.6	4.0	1.30	0.57

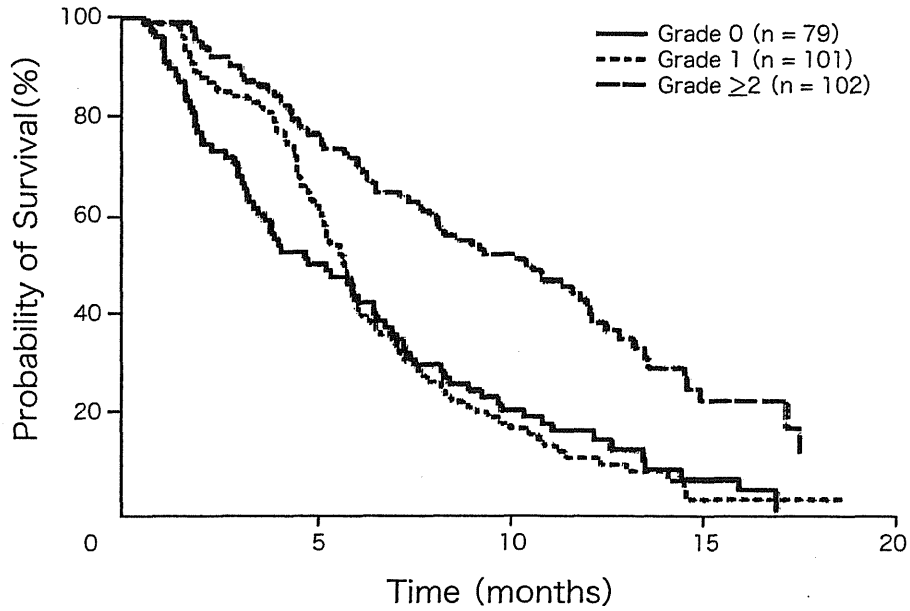

図2　皮疹の程度別の生存曲線

いことから，何らかの人種間格差も考えられている．本結果により現在，本邦でも承認申請中である．

4 治療効果予測因子

EGFR-TKIや抗EGFR抗体薬の治療効果予測因子(バイオマーカー)に関する研究が盛んに行われている．非小細胞肺癌ではEGFR遺伝子変異の有無とEGFR-TKIによる治療効果との強い相関が報告されている[25]．また，結腸・直腸癌においてEGFRの下流のシグナルであるK-RASに遺伝子変異を有する症例では，抗EGFR抗体薬であるcetuximabの効果は期待できないことが報告されている[26]．

PA.3試験ではK-RAS遺伝子変異が判定可能であった117例のうち92例(79％)が変異型であり，25例(21％)が野生型であった．それぞれについてサブグループ解析がなされているが(表)，K-RAS遺伝子変異の有無がerlotinibによる生存延長の予測因子になるという結論には至っていない[27,28]．

またGEM＋erlotinib群において副作用の皮疹の程度がgrade 1以下の患者群のMSTが約5カ月であるのに対し，grade 2以上の皮疹が出現した患者群のMSTが10.5カ月と明らかな予後の延長を認めており，皮疹の

程度と治療効果が相関することが報告されている(図2). JO20302試験においても皮疹がgrade1以下の患者群ではMSTが8.31カ月であるのに対して，grade 2以上の患者群でのMSTは10.25カ月と同様の傾向が認められた．海外において，erlotinibの効果と皮疹の関係を検討する目的で，皮疹が出現するまでerlotinibを増量する試みもされている．主にGEM不応後の51例を対象にerlotinib単剤を投与し，2週間ごとの評価にて皮疹が出現するまで増量(最大250 mg/ Day)する第II相試験が行われた．Grade 2以上の皮疹が出現した症例で病勢制御が良好であったと報告されている[29]．これまでの報告では，erlotinib投与後の皮疹の発現状況が効果予測因子のひとつと考えられており，皮疹の発現が予測できれば，より効果的である．効果予測因子の検討は，乳癌や非小細胞肺癌では，pAktやEGFR intron1などがその候補として報告されている[30,31]．海外では，膵癌を対象に皮疹の発現予測や他のバイオマーカーを検討する目的で2つの臨床試験[32,33]が進行中であり，これらの結果が待ち望まれる．

5 おわりに

進行膵癌においてGEM + erlotinib併用療法の有用性が示されているが，これまでの臨床成績から，より有用なバイオマーカーの探索が今後の重要な課題であると考えられる．

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Clin J Gastroenterol (2011) 4:323–330
DOI 10.1007/s12328-011-0242-9

CASE REPORT

Carcinoid tumor of the gallbladder: report of two cases

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Received: 27 March 2011 / Accepted: 14 June 2011 / Published online: 23 July 2011

Abstract We report two cases of carcinoid tumor of the gallbladder. Case 1 was a 59-year-old woman who presented with epigastric pain. Abdominal ultrasonography and computed tomography (CT) revealed a 16 mm polypoid lesion in the neck of the gallbladder. Tumor markers were within normal limits. Open cholecystectomy was performed with a preoperative diagnosis of early cancer of the gallbladder. Case 2 was a 45-year-old man. A polyp in the gallbladder was incidentally detected on annual checkup. Ultrasound and CT showed an 18 mm protruding lesion in the neck of the gallbladder. Laparoscopic cholecystectomy was performed and the tumor diagnosed as a carcinoid tumor based on the findings of funicular and tubular cells in the lamina propria mucosa, homogeneous nuclei, basophilic cytoplasm, and positive staining with chromogranin A and synaptophysin. The postoperative course of both patients was uneventful, with no recurrence at 44 and 41 months after surgery. In this literature review of 39 cases, classical carcinoid of the gallbladder has a favorable postoperative outcome. Of cases reviewed, 60% are located in the neck of the gallbladder and 50% have a polypoid shape.

Keywords Carcinoid tumor · Gallbladder · Neuroendocrine cell carcinoma

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Introduction

Carcinoid tumor of the gallbladder is extremely rare representing about 0.2% of all carcinoid lesions [1]. Among polypoid lesions of the gallbladder, cholesterol polyps are the most common (62.8%) while carcinoid tumors are extremely rare and included among miscellaneous tumors [2]. Carcinoid tumors are classified into two groups, classical carcinoid tumors with a good prognosis and slow progress, and endocrine cell carcinomas with a poor prognosis and rapid progress. Carcinoid tumors arise mainly in the gastrointestinal tract or the bronchi, and rarely develop in the gallbladder [1]. Carcinoid tumors have been reported more frequently, partly because of progress in various diagnostic modalities. This report describes classical carcinoid tumors found in the gallbladder with a comprehensive review of the relevant literature.

Case report 1

The patient was a 59-year-old woman with epigastric pain. A polyp in the gallbladder was seen on abdominal ultrasonography (US) (Fig. 1). Abdominal computed tomography (CT) showed slight enhancement of the tumor (Fig. 2a, b). Drip infusion cholangiography CT revealed a 16 mm polypoid lesion in the neck of the gallbladder (Fig. 2c). Tumor markers including carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) were within normal limits. Open cholecystectomy was performed with a preoperative diagnosis of early cancer of the gallbladder. Since intraoperative pathological diagnosis also showed cancer of the gallbladder, lymph node dissection was added to the procedure (Fig. 3). Final histopathological diagnosis

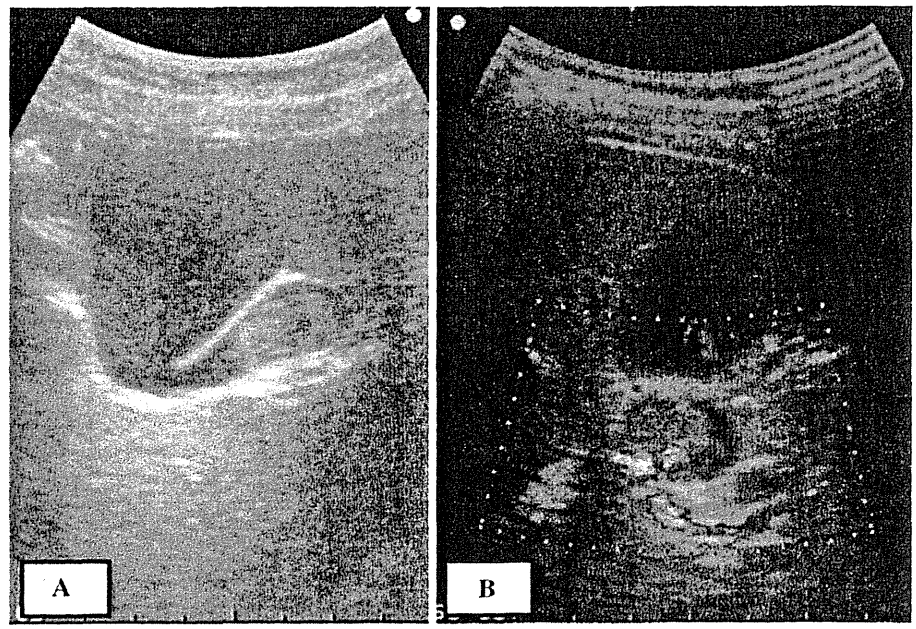

Fig. 1 A 16 mm tumor is shown at the neck of the gallbladder on abdominal US in patient 1 (**a** B-mode, **b** color Doppler)

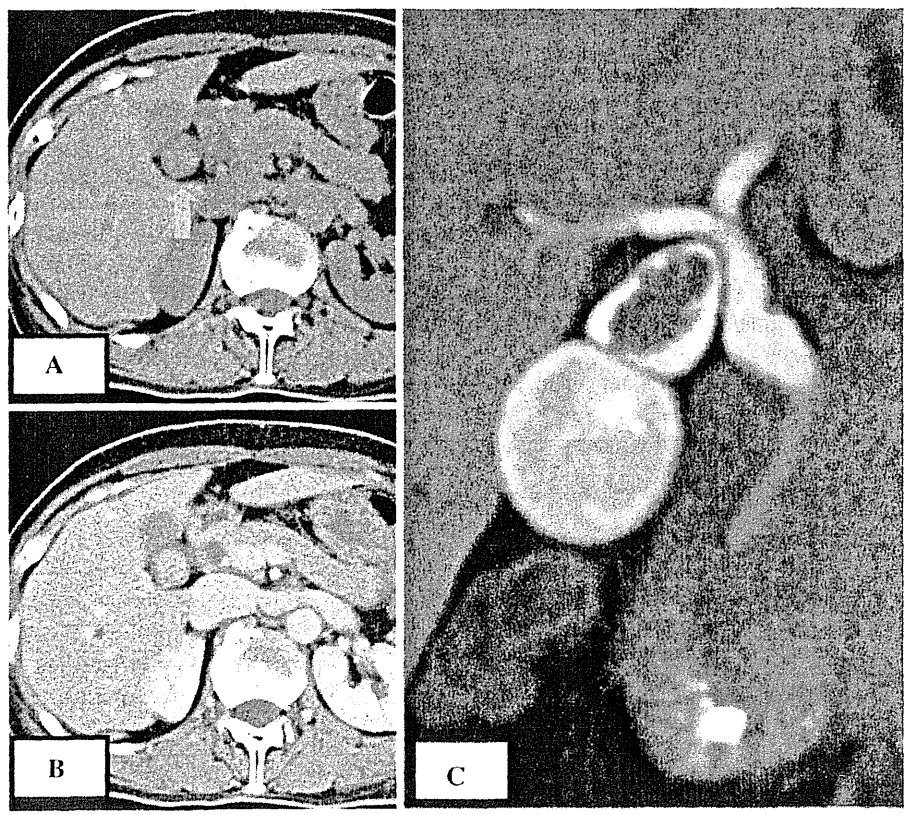

Fig. 2 A 16 mm tumor (*arrow*) is shown at the neck of the gallbladder on abdominal CT (**a** plain, **b** enhanced phase) and drip infusion cholangiography CT (**c** coronal reconstruction) in patient 1

revealed that the lesion was a carcinoid tumor of the gallbladder, based on the findings of funicular and tubular cells in the lamina propria mucosa, homogeneous nuclei, basophilic cytoplasm (Fig. 4a, b), and positive staining for chromogranin A (Fig. 4c), synaptophysin (Fig. 4d), cytokeratin (Fig. 4e) and MIB-1 (Fig. 4f). The tumor was

localized in the mucosa, and no lymph duct or venous invasion were observed. The patient was discharged uneventfully on the eighth postoperative day, and showed no recurrence at 44 months postoperatively.

Case report 2

The patient was a 45-year-old man without symptoms. A polyp in the gallbladder was incidentally observed during annual abdominal US screening (Fig. 5a). Endoscopic US revealed an 18 mm protruding lesion in the neck of the gallbladder (Fig. 5b). Abdominal CT showed slight enhancement of the tumor (Fig. 6a, b). Drip infusion cholangiography CT revealed an 18 mm polypoid lesion in the neck of the gallbladder (Fig. 6c). Tumor markers such as CEA and CA19-9 were within normal limits. Laparoscopic cholecystectomy was performed with a preoperative diagnosis of a benign lesion (e.g., cholesterol polyp) (Fig. 7). Postoperative histopathological analysis showed a carcinoid tumor of the gallbladder based on the findings of funicular and tubular cells in the lamina propria mucosa, homogeneous nuclei, basophilic cytoplasm (Fig. 8a, b), and positive staining for chromogranin A (Fig. 8c), synaptophysin (Fig. 8d), S-100 (Fig. 8e) and CD56 (Fig. 8f). The patient was discharged uneventfully on the fourth postoperative day, and showed no recurrence at 41 months postoperatively.

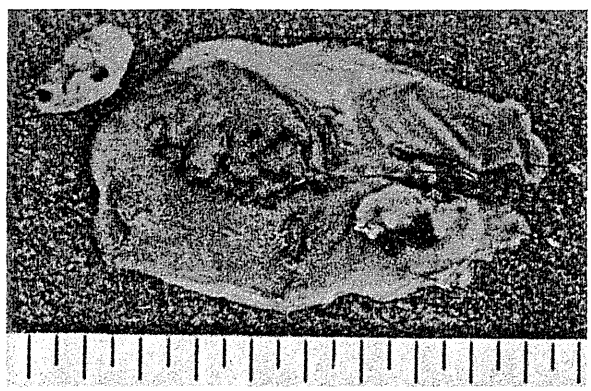

Fig. 3 Resected specimen in patient 1 shows a polypoid tumor at the neck of the gallbladder

Discussion

Here we report two cases of carcinoid tumors of the gallbladder. Carcinoid tumor of the gallbladder was first reported by Joel in 1929 [3] and first reported in Japan by Funabashi et al. [4] in 1978. Carcinoid tumors, previously a generic term for endocrine tumors, are classified into two categories, classical carcinoid tumors and endocrine cell carcinomas [4–6]. There remains confusion about the definition of carcinoid tumors, which has led to a poor understanding of these lesions. Some investigators have described neuroendocrine cell carcinomas of the

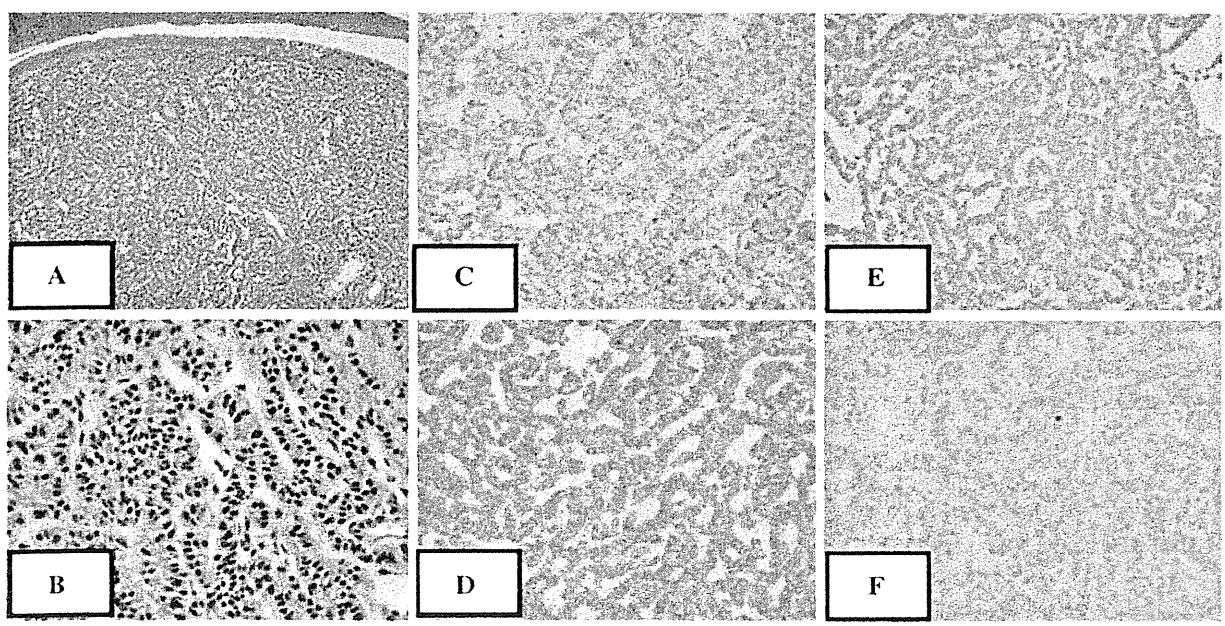

Fig. 4 Histopathological findings of the resected specimen show a carcinoid tumor on H&E staining (**a** ×200, **b** ×400) and immunochemical staining for chromogranin A (**c** ×400), synaptophysin (**d** ×400), cytokeratin (**e** ×400) and MIB-1 (**f** ×400) in patient 1

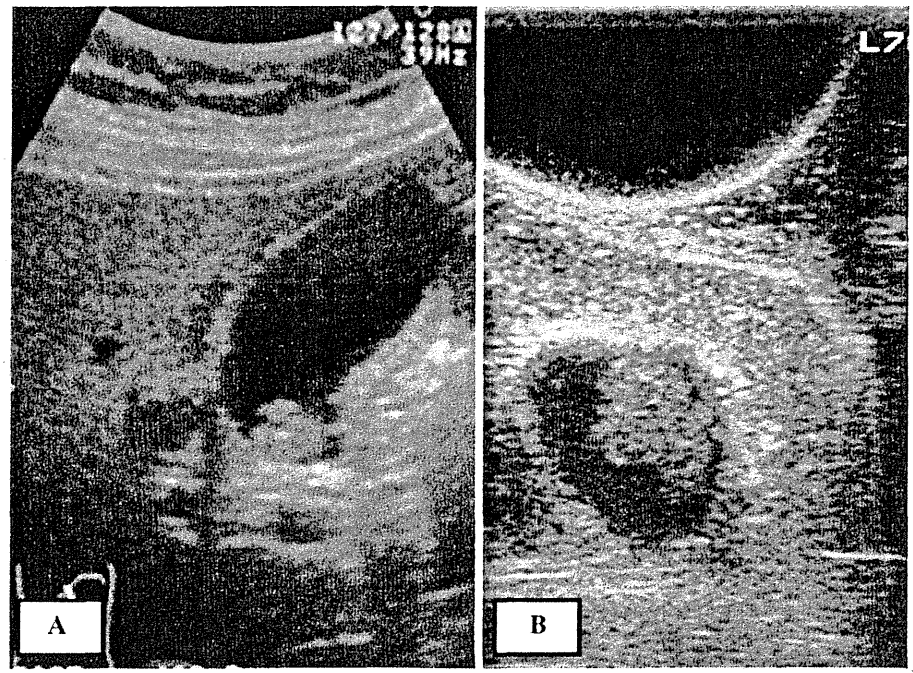

Fig. 5 An 18 mm tumor at the neck of the gallbladder on abdominal US (**a**) and endoscopic US (**b**) in patient 2

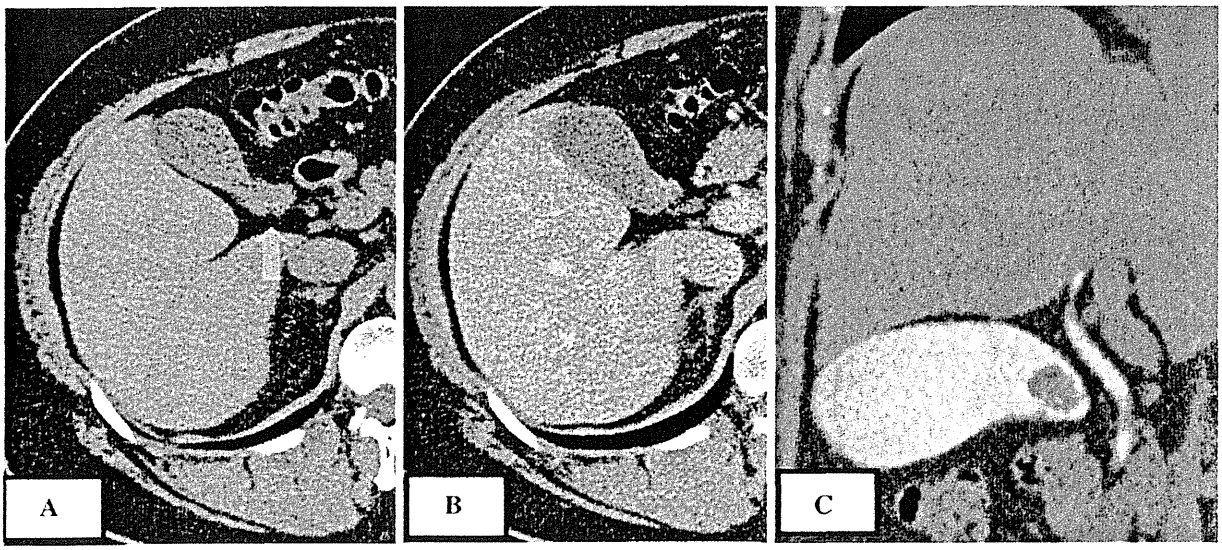

Fig. 6 An 18 mm tumor (*arrow*) at the neck of the gallbladder on abdominal CT (**a** plain, **b** enhanced phase) and DIC-CT (**c** coronal reconstruction) in patient 2

gallbladder as carcinoid tumors even though they show a distinctively different histological form and natural history [5, 6]. Modlin et al. [7] pointed out that half of the previously reported cases of carcinoid tumor of the gallbladder should actually be classified as neuroendocrine carcinomas. Ishikawa et al. [4] discussed classification from the viewpoint of histopathological findings, and found that 25 out of 35 reported cases were classical carcinoid tumors and 10 cases were neuroendocrine carcinomas. Mizukami et al. [8] showed that 24 out of 34 reported cases were endocrine cell carcinoma and 10 were classical carcinoid tumors. Zou et al. [9] recently summarized 47 cases in the English literature. Of these 47 cases, there were 27 definite endocrine cell carcinomas and 12 classical carcinoid tumors.

Classical carcinoid tumor and endocrine cell carcinoma were misclassified. In the literature, 37 cases from 26 reports were identified using reviews of the Japana Centra Revuo Medicina and Pub-Med from 1983 to 2010 [4–6, 9–31] (Table 1). In 39 cases, including, the two present cases, 60.5% of carcinoid tumors were located in the neck of the gallbladder. In addition, 50.0% had a polypoid or pedunculated shape macroscopically, and 52.6% showed coexistent cholecystolithiasis. No patient presented with classic carcinoid syndrome. Diagnosis was difficult preoperatively because there is no specific feature of protruding tumors with carcinoid tumors of the gallbladder. Of these patients, 31.6% were diagnosed as having a benign polyp, 31.6% with cancer, and 28.9% as gallstones with Mirizzi syndrome before operation. Open cholecystectomies were performed in most cases and resection of the gallbladder bed or hepatectomy and/or regional lymph node dissection were added in advanced cases. In 1992, Porter et al. [19] first reported laparoscopic cholecystectomy for patients with carcinoid tumors of the gallbladder. Of the patients reviewed, 71.1% showed invasion to the muscle layer or deeper and 21.1% were limited to the mucosa. Recently, the number of cases limited to the mucosa and treated laparoscopically is increasing, because of progress in preoperative imaging [4, 13]. Of those, 65.8% reported cases showed no recurrence after resection. The average disease-free interval after operation is 28.7 months (ranging from 6 to 180 months) in patients who survived and 19.8 months (ranging from 3 to 81 months) in patients who died. Resected carcinoid tumors of the gallbladder are expected to have a good prognosis.

In conclusion, based on this literature review of 39 cases, classical carcinoid of the gallbladder has a favorable postoperative outcome. Of cases reviewed, 60% are located in the neck of the gallbladder and 50% have a polypoid shape.

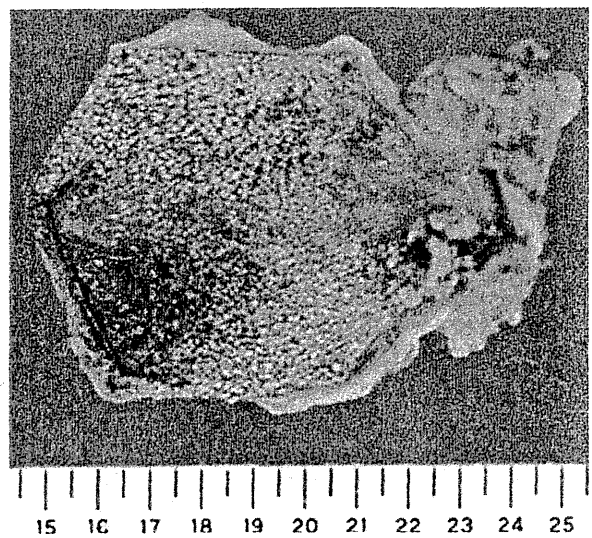

Fig. 7 Resected specimen in patient 2 shows a polypoid tumor at the neck of the gallbladder

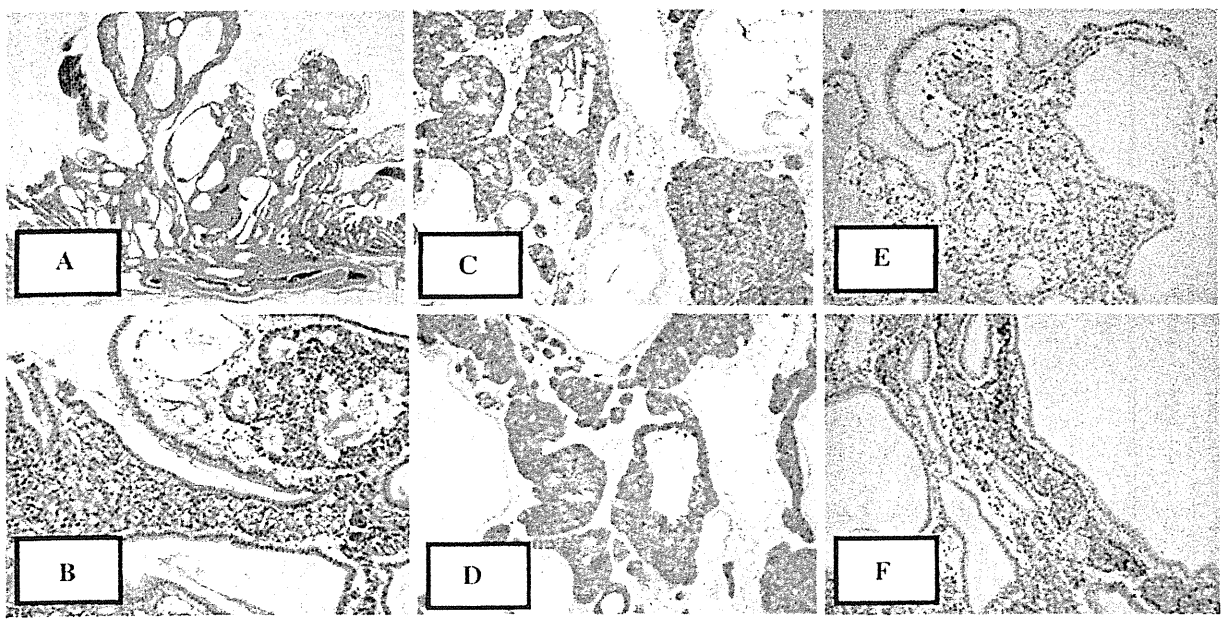

Fig. 8 Histopathological findings of the resected specimen show a carcinoid tumor on H&E staining (**a** ×20, **b** ×200) and immunochemical staining for chromogranin A (**c** ×200), synaptophysin (**d** ×200), S-100 (**e** ×200) and CD56 (**f** ×200) in patient 2

Table 1 Previously reported cases of carcinoid tumors of the gallbladder

No.	Author	Year	Age	Gender	Size (mm)	Macroscopic feature	Location	Stone	Carcionid syndrome	Coexisting GB ca	Depth	T (UICC)	Metastasis	Preoperative diagnosis	Operative procedure	Outcome	
1	Funabashi [4]	1976	28	F	Child head	Mass	NA	−	−	−	hinf+	3	−	GB tumor	OC	4M	Death
2	Takahashi [4]	1978	62	F	80 × 80 × 50	Mass	NA	+	−	−	hinf+	3	LN	GB cancer	OC + LB + LN	4M	Death
3	Amano [4]	1979	62	F	40 × 30 × 20	Elevated lesion	F	+	−	−	hinf+	3	−	GB stone	OC	NA	
4	Haratake [4]	1980	60	F	35 × 30 × 20	Polypoid	NA	+	−	−	hinf+	3	Liver, LN	GB cancer	Autopsy	3M	Death
5	Muto [10]	1984	80	M	10 × 7	Elevated lesion	F	−	−	+	SS	2	−	GB cancer	OC + LB + LN	NA	
6	Kijima [4]	1985	73	F	2 × 2	Polyp	N	NA	NA	−	m	1a	−	NA	NA	NA	
7	Kiiima [4]	1985	44	F	3 × 2	Polyp	N	NA	NA	−	m	1a	−	NA	NA	NA	
8	Kitagawa [11]	1986	64	F	25 × 25 × 20	Polypoid	F	+	−	−	hinf+	3	Liver	GB cancer	Hr (AM) + OC	NA	
9	Katoh [12]	1986	45	M	6 × 4 × 2	Polyp	N	−	−	−	SS	2	−	GB polyp	OC	24M	Alive
10	Tsuge [13]	1987	67	M	25 × 25	Polypoid	B	+	−	−	NA		−	Mirizzi syndrome	OC	18M	Alive
11	Fukunaga [14]	1990	35	F	15 × 9	Polyp	N	+	−	−	SS	2	−	GB stone	OC	13M	Alive
12	Mochizuki [15]	1991	77	M	5 × 3 × 2	Sessile	N	+	−	−	m	1a	−	GB stone	OC	23M	Alive
13	Yoshizumi [16]	1992	49	M	75 × 50 × 40	NA	N + B + F	−	−	−	hinf+	3	LN	GB tumor	Hr (AP) + PD + OC + LN	7M	Death
14	Takeuchi [17]	1992	40	M	15 × 10	Polyp	N	−	−	−	mp	1b	−	GB polyp	OC	6M	Alive
15	Tanaka [18]	1992	62	F	10 × 8 × 3	Pedunculated polyp	N	−	−	−	mp	1b	−	GB polyp	OC	18M	Alive
16	Porter [19]	1992	75	F	30 × 20	Mass	F	−	−	−	SS	2	−	NA	Lap-C	12M	Alive
17	Naseer [20]	1992	60	F	15 × 10 × 5	Pedunculated polyp	N	+	−	−	NA		−	GB stone	OC	NA	
18	Barone [21]	1992	56	M	6	Polyp	N	+	−	−	mp	1b	−	GB stone	OC	12M	Alive
19	Ishida [13]	1993	46	F	5	Flat elevation	F	+	−	−	SS	2	−	GB stone	OC	42M	Alive
20	Deehan [22]	1993	51	F	2.6	Flat elevation	N	+	−	−	mp	1b	−	GB stone	OC	6M	Alive
21	Deehan [22]	1993	33	F	7	Polyp	N	+	−	−	mp	1b	−	GB stone	OC	180M	Alive
22	Sugamura [23]	1996	63	F	25 × 15	Polypoid	B + F	+	−	+	SS	2	Liver	GB cancer	OC + LB + LN	9M	Alive
23	Satoh [24]	1996	70	F	10 × 7	Flat elevation	F	−	−	+	mp	1b	−	GB cancer	OC + LN	10M	Alive
24	Nishigami [5]	1996	41	F	11 × 17	Polyp	N	+	−	−	mp	1b	−	GB polyp	OC	11M	Alive
25	Matsumura [25]	1998	79	F	91 × 81	Mass	B + F	+	−	−	m	1a	−	GB tumor	OC	20M	Alive
26	Suzuki [26]	1999	83	M	4	Elevated lesion	N	+	−	−	mp	1b	−	GB + CBD stone	OC	11M	Alive

Table 1 continued

No.	Author	Year	Age	Gender	Size (mm)	Macroscopic feature	Location	Stone	Carcionid syndrome	Coexisting GB ca	Depth	T (UICC)	Metastasis	Preoperative diagnosis	Operative procedure	Outcome	
27	Kaiho [6]	1999	58	M	26 × 15	Pedunculated polyp	N	−	−	−	mp	1b	−	GB cancer	OC + BD + LN	30M	Alive
28	Hibono [27]	2002	61	F	70 × 45	Mass	N + B + F	+	−	−	hinf+	3	−	GB cancer	Hr (APM)+OC+LN	81M	Death
29	Kinugasa [28]	2004	41	F	11 × 7	Papillary	N	+	−	−	mp	1b	−	GB polyp	Lap-C	102M	Alive
30	Kita [29]	2005	50	F	10 × 7	Polypoid	N	−	−	−	mp	1b	LN	GB tumor	OC + LN	12M	Alive
31	Sato [13]	2006	66	M	12 × 7.5	Papillary	N	−	−	−	SS	2	−	Cholesterol polyp	Lap-C	44M	Alive
32	Ishikawa [4]	2006	64	F	12	Polypoid	N	+	−	−	m	1a	−	GB polyp	Lap-C	12M	Alive
33	Geo [30]	2007	52	M	NA	Wall thickness	NA	+	−	−	NA		−	GB stone	OC	NA	
34	Anianevulu [31]	2007	53	F	50 × 45 × 35	Mass	B + F	−	−	−	SS	2	−	GB cancer	OC	NA	
35	Oku [13]	2008	64	M	14 × 8	Pedunculated polyp	N	−	−	−	m	1a	−	GB cancer	Lap-C	6M	Alive
36	Zou [9]	2010	46	F	40 × 50	Mass	B + F	−	−	−	mp	1b	−	GB cancer	OC	12M	Alive
37	Present case 1	2010	59	F	16	Polypoid	N	−	−	−	m	1a	−	GB cancer	OC	44M	Alive
38	Present case 2	2010	45	M	18	Polypoid	N	−	−	−	m	1a	−	GB polyp	Lap-C	41M	Alive

GB gallbladder, *NA* not available. Location: *N* neck, *B* body, *F* fundus. Metastasis: *LN* lymph node. Operative procedure: *OC* open cholecystectomy, *Lap-C* laparoscopic cholecystectomy, *LB* resection of the liver bed, *LN* dissection of the regional lymph nodes, *Hr* hepatic resection (*A* anterior segment, *P* posterior segment, *M* medial segment), *PD* pancreatoduodenectomy, *BD* bile duct resection, Outcome: *M* month

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Cancer Science
The official journal of the Japanese Cancer Association JCA

Identification of the transforming activity of Indian hedgehog by retroviral expression screening

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(Received May 10, 2009/Revised August 29, 2009/Accepted September 2, 2009/Online publication September 30, 2009)

To identify novel cancer-promoting genes in biliary tract cancer (BTC), we constructed a retroviral cDNA expression library from a clinical specimen of BTC with anomalous pancreaticobiliary duct junction (APBDJ), and used the library for a focus formation assay with 3T3 fibroblasts. One of the cDNAs rescued from transformed foci was found to encode Indian hedgehog homolog (IHH). The oncogenic potential of IHH was confirmed both *in vitro* with the focus formation assay and *in vivo* with a tumorigenicity assay in nude mice. The isolated *IHH* cDNA had no sequence alterations, suggesting that upregulation of IHH expression may contribute to malignant transformation. Quantitation of *IHH* mRNA among clinical specimens has revealed that the expression level of *IHH* in BTC with APBDJ is higher than that in BTC without APBDJ and than in non-cancerous biliary tissues. Our data thus implicate a direct role of IHH in the carcinogenesis of BTC with APBDJ. (*Cancer Sci* 2010; 101: 60–64)

Biliary tract cancer (BTC) is a highly fatal malignancy in humans, and is prevalent in South American and Asian countries; approximately sixteen thousand people die of BTC every year in Japan.[1] Unfortunately, many BTC cases are diagnosed at advanced clinical stages with a 5-year survival rate of ~10%.[2–4] Several risk factors for BTC have been identified to date, including cholelithiasis,[5] anomalous pancreaticobiliary duct junction (APBDJ),[6] and primary sclerosing cholangitis.[7] Genetic alternations in *KRAS* or *TP53* and/or overexpression of *ERBB2* have been shown to contribute to the development of certain types of BTC. However, many cases with BTC do not harbor any such genetic changes, and other transforming events further await discovery.

The focus formation assay with 3T3 or RAT1 fibroblasts has been extensively used to screen for transforming genes in various carcinomas.[8] In such screening, genomic DNA is isolated from cancer specimens, and used to transfect 3T3 fibroblasts to obtain transformed cell foci. As expression of transfected genes in 3T3 cells in this assay is regulated by their own promoter and enhancer fragments, oncogenes with tissue-specific expression (e.g. those with a blood cell-specific promoter) can not become transcriptionally active in 3T3 cells, and thus can no longer be captured in such a screening system.

To ensure the sufficient expression of oncogenes in 3T3 cells, their transcription should be directly regulated by an exogenous promoter fragment. We have therefore constructed a retroviral cDNA expression library from a surgically operated clinical specimen of BTC with APBDJ, which was subsequently used to infect 3T3 cells. In the preparation of the cDNA library, we further took advantage of the SMART PCR system (Clontech, Mountain View, CA, USA), which preferentially amplifies full-length cDNA. A focus formation assay with the library has resulted in the identification of a transforming Indian hedgehog homolog (*IHH*) cDNA.

Materials and Methods

Focus formation assay with a retroviral library. A recombinant retroviral library was constructed as described previously,[9–12] with minor modifications. In brief, total RNA was extracted from a BTC specimen with APBDJ isolated from a 67-year-old man, who gave informed consent. This study was approved by the ethics committee of Jichi Medical University. 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). The resulting cDNA was then amplified by PCR with 5′-PCR primer IIA (Clontech) and PrimeSTAR HS DNA polymerase (Takara Bio, Shiga, Japan) for 18 cycles of 98°C for 10 s and 68°C for 6 min. The PCR products were ligated to a *Bst*XI adapter (Invitrogen, Carlsbad, CA, USA) and then incorporated into the pMXS retroviral plasmid (kindly provided by T. Kitamura of the Institute of Medical Science, University of Tokyo). A total of 5.8×10^5 colony forming units of independent plasmid clones was thus generated. Twenty clones were randomly isolated from the library, and examined for the incorporated cDNA. Sixteen (80%) out of the 20 clones contained cDNA inserts with an average length of 1.16 kbp. Recombinant retroviruses were produced by introduction of the plasmid library into the packaging cell line BOSC23 (American Type Culture Collection, Manassas, VA, USA) and were used to infect 3T3 cells in the presence of 4 μg/mL polybrene (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 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.

Tumorigenicity assay in nude mice. 3T3 cells (2×10^6) were infected with a retrovirus expressing IHH, resuspended in 500 μL PBS, and injected into each shoulder of a *nu/nu* Balb-c mouse (6 weeks old). Tumor formation was assessed after 2 weeks.

Anchorage-independent growth in soft agar. 3T3 cells (2×10^6) were infected with a retrovirus encoding IHH or v-Ras, resuspended in the culture medium supplemented with 0.4% agar (Sea Plaque GTG agarose; Cambrex, East Rutherford, NJ, USA), and seeded onto a base layer of complete medium supplemented with 0.5% agar. Cell growth was assessed after culture for 2–3 weeks.

Quantitative RT-PCR analysis. Portions of oligo(dT)-primed cDNA produced by reverse transcription were subjected to PCR with a QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA, USA) and an amplification protocol comprising incubation at 94°C for 15 s, 60°C for 30 s, and 72°C for 60 s. Incorporation

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doi: 10.1111/j.1349-7006.2009.01355.x

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 PCR products begins. The C_T values for cDNA corresponding to the β-actin gene (*ACTB*) and *IHH* 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′-CCTCTCCTAGAGACCTTG-3′ and 5′-CTGGCTCCCAGGGAATTTAG-3′ for *IHH*.

Immunohistochemistry. Human tissues were fixed in 4% formaldehyde in PBS overnight at room temperature, embedded in paraffin, and sectioned at a thickness of 3 μm. Sections were mounted on glass slides, deparaffinized in 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. 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 overnight at 4°C with anti-IHH antibody (sc-1196; Santa Cruz Biochemistry, Santa Cruz, CA, USA) diluted in PBS. Next, the sections were washed in PBS and incubated with horseradish peroxidase-labeled polymers conjugated to secondary antibodies for primary rabbit antigoat immunoglobulin (Dako, #P0449) without dilution 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 the chromogenic substrate. Finally, the sections were lightly counterstained with hematoxylin, mounted, and viewed under a light microscope. For the negative control, the immunostaining processes were carried out by replacing the primary antibody with PBS.

Results

Screening with the focus formation assay. From the mRNA of a BTC specimen with APBDJ, full-length cDNA was selectively amplified and ligated to a retroviral vector pMXS. From such library plasmids, we generated a recombinant ecotropic retrovirus that was subsequently used to infect mouse 3T3 fibroblasts. Infection experiments were repeated for a total of four times. After 3 weeks of culture, 75 transformed foci were observed. No foci could be found among the cells infected with an empty virus, while numerous foci were easily identified in the cells infected with a virus expressing v-Ras oncoprotein (data not shown).

Table 1. Bile duct cancer cDNA isolated from 3T3 transformants

Clone ID #	Gene symbol	GenBank no.	Presence of full ORF
1	*FAM83H*	NM_198488	No
2	*GATAD1*	NM_021167	Yes
3	*RRAS2*	NM_012250	No
4	*FASTK*	NM_006712	Yes
5	*VAT1*	NM_006373	Yes
6	*ARPC2*	NM_005731	No
7	*IHH*	NM_002181	Yes
8	*SENP6*	NM_015571	Yes
9	*DOT1L*	NM_032482	ND
10	*LTBR*	NM_002342	ND
11	*KRAS*	NM_004985	Yes
12	*TMEM54*	NM_033504	Yes
13	*RNASET2*	NM_003730	Yes
14	*RPS4X*	NM_001007	Yes
15	*TETRAN*	NM_001120	Yes
16	*DFNB31*	NM_015404	No
17	*CLDN3*	NM_001306	No
18	*GJB2*	NM_004004	Yes
19	*PSMA7*	NM_002792	Yes
20	*PRPSAP1*	NM_002766	Yes
21	*LRRC59*	NM_018509	Yes
22	*LRP5*	NM_002335	ND
23	*NCOR2*	NM_006312	No
24	*KLF16*	NM_031918	No
25	*ARHGAP4*	NM_001666	ND
26	*KIAA0284*	NM_015005	No
27	*DNAJC4*	NM_005528	ND
28	*NOTCH2NL*	NM_203458	No
29	*BCKDHB*	NM_000056	Yes

ND, not determined; ORF, open reading frame.

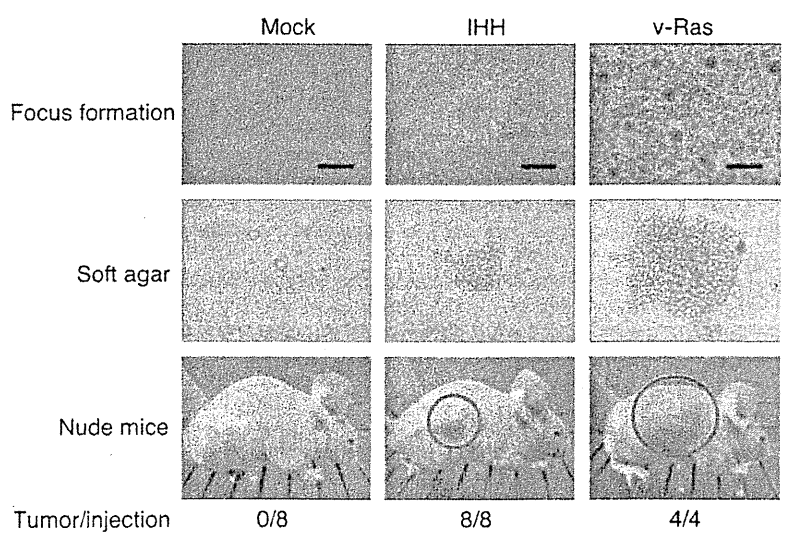

Fig. 1. Transforming activity of Indian hedgehog homolog (IHH). Mouse 3T3 cells were infected with viruses encoding IHH or v-Ras or with the empty virus (Mock), and were then cultured for 5 days for the analysis of focus formation (top panels; scale bars = 1 mm). The same batches of 3T3 cells were also 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 (tumors/injection) is also indicated.

Each focus was isolated, expanded independently, and used to prepare genomic DNA. We then tried to recover retroviral inserts from such genomic DNA by PCR amplification with the primer used originally to amplify the cDNA in the construction of the library. In most cases, one to three DNA fragments were recovered from each genome, implying multiple retroviral infection of some 3T3 cells.

We finally obtained a total of 44 cDNA fragments by PCR, each of which was ligated into a cloning vector, and subjected to nucleotide sequencing from both ends. Screening of the 44 cDNA sequences against the public nucleotide sequence databases revealed that the 44 fragments correspond to 29 independent genes (Table 1).

Identification of IHH. To confirm the transforming potential of the isolated cDNA, each cDNA clone was ligated to pMXS, and corresponding retrovirus was used to re-infect 3T3 cells. Focus formation assays were conducted for 13 independent genes, discovering a reproducible transforming activity for clone ID #7 corresponding to *IHH* (GenBank accession number, NM_002181) (Fig. 1, top panel). Again, infection with a virus for v-Ras induced many transformed foci, while an empty virus failed to do so. The entire coding region of our ID #7 cDNA was sequenced, revealing no point mutations or deletions compared to the published *IHH* cDNA sequence. Although activation of Hedgehog (Hh) pathways has been revealed among a wide range of digestive tract cancers,[13] oncogenic activity of *IHH* has not been reported to date. We supposed from our data that overexpression of *IHH* may contribute directly to malignant transformation.

Confirmation of the transforming activity of IHH. To confirm the oncogenic activity of IHH, we examined its effect on the anchorage-independent growth of 3T3 cells in soft agar. Whereas cells infected with an empty virus did not grow in the agar, those infected with a virus expressing IHH formed multiple foci in repeated experiments (Fig. 1, middle panel). In addition, 3T3 cells expressing v-Ras readily grew in the agar.

The transforming activity of IHH was also tested by the tumor formation assay with athymic nude mice. 3T3 cells infected with the empty virus or retrovirus expressing IHH or v-Ras were inoculated subcutaneously into nude mice. As shown in the bottom panel of Fig. 1, tumor formation was readily observed for the cells expressing IHH or v-Ras. These results clearly revealed

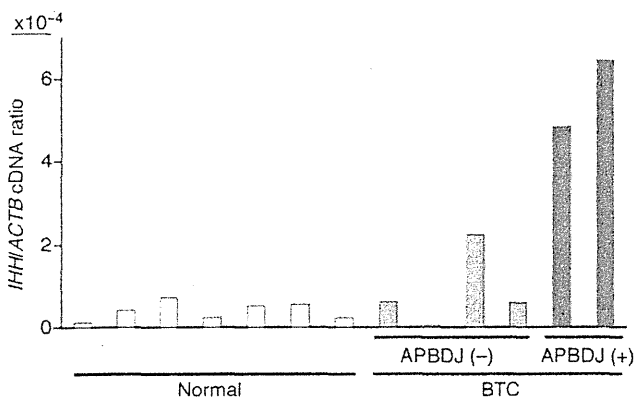

Fig. 2. Expression of Indian hedgehog homolog (*IHH*) in biliary tract. Oligo(dT)-primed cDNA was synthesized from clinical specimens of biliary tract cancer (BTC) with (+) or without (–) anomalous pancreaticobiliary duct junction (APBDJ), or from normal gallbladder (Normal), and were subjected to quantitative PCR analysis for cDNA of *IHH* and β-actin (*ACTB*). The relative expression level of the former to the latter is represented.

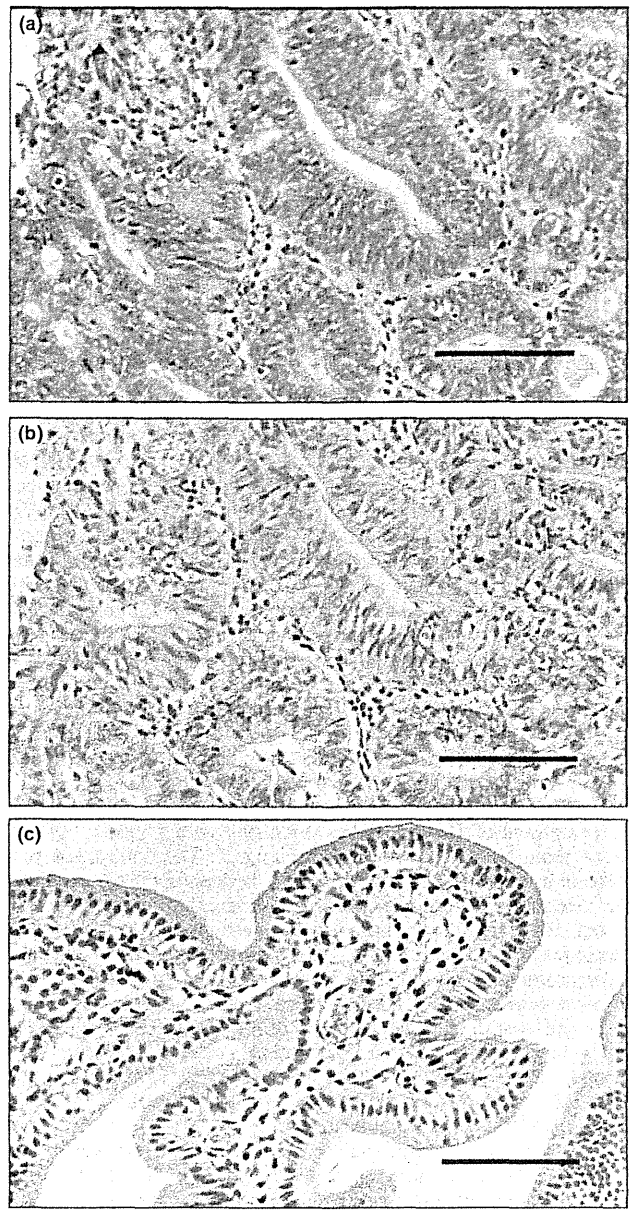

Fig. 3. Immunohistochemical detection of Indian hedgehog homolog (IHH). Expression of IHH is elevated in (a) biliary tract cancer with anomalous pancreaticobiliary duct junction, but such reactivity was absent in the control experiment for (b) the same specimen or (c) anti-IHH staining in normal gallbladder. Scale bars = 100 μm.

an unexpected, direct transforming potential of IHH in fibroblasts.

Overexpression of *IHH* mRNA. Given the transforming potential of wild-type IHH (when it is abundantly expressed), we tried to examine if *IHH* is overexpressed in BTC specimens. Real-time RT-PCR analysis for the quantitation of *IHH* cDNA among normal gall bladder (n = 7) and BTC specimens (n = 6) (Supporting Information Table S1) revealed that *IHH* is indeed overexpressed in the latter specimens, albeit with marginal statistical significance (P = 0.06) by a two-tailed t-test (Fig. 2). It should be noted, however, that BTC cases with APBDJ (n = 2) had significantly abundant expression of *IHH* compared to BTC

doi: 10.1111/j.1349-7006.2009.01355.x

without APBDJ ($P = 0.005$) or to normal gall bladder ($P = 2.4 \times 10^{-6}$). Therefore, it is likely that some types of BTC overexpress IHH.

Protein expression of IHH. To confirm the elevated expression of IHH in BTC, we examined its protein level by an immunohistochemical approach. In accordance with the RT-PCR experiments, IHH protein was abundantly detected only in the cytoplasm of cancerous duct but not in stromal cells for BTC with APBDJ (Fig. 3). We failed to observe such staining in normal gallbladder, suggesting that IHH protein was markedly induced in BTC with APBDJ compared to normal gallbladder.

Discussion

In the present study, we have constructed a retroviral cDNA expression library for a BTC specimen with APBDJ, and unexpectedly revealed the transforming potential of IHH through a focus formation assay with the mouse fibroblast cell line 3T3. As there were no sequence alterations in our isolated *IHH* cDNA, the high expression of IHH is likely to exert its oncogenic activity. Consistent with this notion, expression of *IHH* was indeed activated in BTC with APBDJ.

In our transformation assays for IHH (i.e. focus formation assay, soft agar-growth assay, and nude mouse-tumorigenicity assay) we directly used a highly polyclonal, mass culture of 3T3 cells infected with a retrovirus expressing IHH, without any selection (such as positive selection for neomycin resistance-cells). Repeated confirmation of the transforming potential for IHH in such assays (and not for an empty virus) strongly argues against a hypothesis that an artificial expression of mouse genes adjacent to the retroviral integration sites was responsible for the 3T3 transformation in these experiments.

The Hh signaling pathway was originally described in the development of *Drosophila melanogaster* as a segment polarity gene required for embryonic patterning.[14] There are three vertebrate homologues of Hh: Ihh, Sonic hedgehog (Shh), and Desert hedgehog (Dhh) with similar biological properties among them. Hh signaling is known to play a pivotal role in cell fate decisions,[15] tissue repair,[16] and stem cell self renewal.[17,18] Aberration in such signaling may contribute to sustained cell growth and cancer. Indeed, Hahn *et al.* and Johnson *et al.* revealed that mutations within *PTCH1* (a binding partner of hedgehog) cause a cancer-promoting condition, Gorlin syndrome.[19,20] Further, frequent mutations in Hh signaling components have also been identified among sporadic basal cell carcinoma[21] and medulloblastoma.[22]

In addition, transcriptional activation of Hh components has been demonstrated among a wide range of gastrointestinal tumors, which results from endogenous overexpression of Hh proteins such as IHH and SHH.[13] Despite the lack of gene mutations for the Hh components in these tumors, cyclopamine, a specific inhibitor for SMO, suppresses the growth of tumors positive for elevated Hh signaling, supporting the idea that overexpression of the Hh family of proteins may have a mitogenic function.

Our current data proves for the first time the direct transforming potential of IHH, at least in fibroblasts. Furthermore, apparent overexpression of *IHH* in BTC with APBDJ indicates an important role of IHH especially in this subtype of BTC. In addition to the presence or absence of APBDJ, we also examined the clinicopathological features of the BTC specimens used in our study. As shown in Supporting Information Table S1, none of the TNM stage, clinical stage, KRAS mutation, or Ki-67 index were related to the overexpression of *IHH*. However, because the current cohort size is still small, a larger cohort study is mandatory to examine the clinical features of BTC with high *IHH*.

Although Yang *et al.* reported that treatment with a SMO inhibitor leads to downregulation of *CCND1* and upregulation of *CDKN1A* in a cell line of pancreatic carcinoma,[23] we did not observe such a relationship between *CCND1/CDKN1A* and *IHH* expression (data not shown). However, overexpression of *CCND1* may be more prevalent among BTC than that of *IHH*,[24] suggesting the presence of an IHH-independent regulatory network for *CCND1* in BTC.

APBDJ causes pancreatic fluid regurgitation into the biliary duct, and is found frequently among BTC cases.[25] Because pancreatic fluid is rich in various proteases, frequent regurgitation of such fluid into the biliary tract is likely to cause sustained inflammation in the tract. Because inflammation and tissue repair cause transcriptional activation of the Hh family of soluble factors,[16] it may not be surprising to find an elevated level of *IHH* mRNA in the biliary tract with APBDJ. Given the transforming function of abundant IHH, such overexpression may lead to increased cell cycle of biliary tract cells, and eventually to the generation of BTC. Because a number of chemical inhibitors are under development for the Hh pathways,[26] BTC with APBDJ would be an intriguing candidate for such drugs. Further, it is also tempting to examine the Hh ligand levels among human cancers associated with chronic inflammation or regeneration.

Acknowledgments

This work was supported in part by grants for Research on Human Genome and Tissue Engineering and for Third-Term Comprehensive Control Research for Cancer from the Ministry of Health, Labor, and Welfare of Japan, as well as by a grant for Scientific Research on Priority Areas "Applied Genomics" from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Abbreviations

CCND1	cyclin D1
CDKN1A	cyclin-dependent kinase inhibitor 1A
ERBB2	v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2
KRAS	v-ki-ras2 Kirsten rat sarcoma viral oncogene homolog
PTCH1	Patched, Drosophila, homolog of, 1
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. Clinical characteristics of the patients with biliary tract cancer (BTC).

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doi: 10.1111/j.1349-7006.2009.01355.x