cells to the state of EMT. Therefore, we generated the MSX2 stable expressing pancreatic cancer cell lines to assess whether this gene could cause EMT. Our results clearly show that MSX2 led the pancreatic cancer cells to the state of EMT based on the following. 1) MSX2-expressing cells had a more scattered and flattened phenotype with fewer intercellular contacts than the control cells. 2) The localization of E-cadherin and β-catenin was changed from its usual cell membrane-associated site to diffuse distribution in the cytoplasm, and this localization was restored when Panc-1 cells that express very high levels of endogenous MSX2 were stably transfected with MSX2si construct to significantly decrease its expression. 3) The wound healing scratch and the two-chamber migration assays clarified the cell migratory effect of MSX2, and this effect was reversed when MSX2 was down-regulated in Panc-1. 4) Metastases to the liver and disseminations to peritoneum were more frequently demonstrated in the pancreas of the mice implanted with MSX2-expressing cells compared to MSX2 down-regulated cells, and this effect was reversed when MSX2 was down-regulated in Panc-1.

To our knowledge, the involvement of MSX2 in pancreatic cancer has not been clarified previously. MSX2 expression was more intense in pancreatic cancer cell lines examined than normal cells and found in more than 70% of human pancreatic carcinoma tissues, whereas no or very weak expression was detected in normal pancreatic ducts. Interestingly, this expression was associated with less differentiation of carcinoma cells, suggesting that MSX2 is involved mainly in pancreatic carcinoma progression rather than carcinogenesis. MSX2 has been suggested to act to stimulate proliferation and inhibit differentiation of osteoprogenitors.[35] MSX2 also caused an increase in the number of proliferative osteoblasts in the osteogenic front of the skulls of postnatal mice.[36] In addition, MSX2 stimulates branching morphogenesis of mouse mammary ducts,[37,38] indicating that this gene function is associated with the regulation of the differentiation and/or proliferation of epithelial cells as well as osteogenic cells. Furthermore, MSX2 has been shown to be up-regulated in adult pancreas in interferon-γ transgenic mice in which aggressive growth of pancreatic ducts and the continuous differentiation of new endocrine cells were observed,[39] suggesting that MSX2 promotes the growth of duct cells that are the origin of pancreatic cancer. These observations, together with the fact that MSX2 stimulates pancreatic cancer cell proliferation *in vitro*, suggest that MSX2 contributes the development of pancreatic carcinoma by promoting cell proliferation and regulating cellular differentiation.

To clarify the molecular mechanism for poor prognosis of pancreatic cancer patients, we have examined the expression of c-*erb* B-2,[40] gelatinase A,[41] ROCK-1,[42] and survivin[43] and demonstrated that their expression was correlated with the invasiveness and/or the frequency of metastasis in pancreatic cancer. In addition to these factors, we have shown in the current study that MSX2 expression is correlated with biological aggressiveness of human pancreatic cancer. Although up-regulation of MSX2 in carcinoma of epithelial origin has been demonstrated, there was no investigation of the association of MSX2 expression and clinicopathological features of any type of carcinomas. Thus, the current results are the first demonstration that increased MSX2 expression is involved in poor differentiation of carcinoma cells. Although poor differentiation of pancreatic carcinoma is associated with short survival time,[44] further studies in this area are required.

Twist 1 was initially identified as a crucial regulator of embryonic morphogenesis in *Drosophila*.[45] Recent studies reveal that Twist 1 expression is associated with invasion and/or metastasis in breast and nasopharyngeal cancer.[46] Ectopic expression of Twist 1 resulted in loss of E-cadherin-mediated cell adhesion and induction of cell motility, suggesting that this gene promotes an EMT. In pancreatic cancer cells, this gene is shown to be induced when cancer cells are undergoing EMT after vascular endothelial growth factor stimulation.[47] Twist 1 and MSX2 have been reported to control the differentiation and proliferation cooperatively in frontal bone skeletogenic mesenchyme.[48] Although the authors hypothesized either gene could regulate the expression of the other, their results obtained by *in situ* hybridization showed that MSX2 and Twist 1 do not regulate each other's activity at the level of mRNA abundance. Our cDNA array clearly revealed the significant induction of Twist 1 in MSX2 overexpressing BxPC3 cells, and this induction was confirmed by semiquantitative RT-PCR and Western blotting. Conversely, nuclear expression of Twist 1 disappeared when MSX2 was down-regulated in Panc-1 cells. Finally, immunohistochemical analyses revealed that Twist 1 expression was correlated with MSX2 expression in human pancreatic carcinoma tissues, and colocalization of these proteins was demonstrated by double staining of fluorescence immunohistochemistry, indicating that Twist 1 was a target gene of MSX2. Consistent with these findings, MSX2 appears to function in leading the pancreatic cancer cells to the state of EMT and an enhanced malignant phenotype through up-regulation of Twist 1.

Among approximately 55,000 genes, Twist 1 was identified as the gene most up-regulated by MSX2. Although we focused on Twist 1 in this study, since it is an EMT-related gene, we could also identify other candidate target genes for MSX2 by this method. These include the ATP-binding cassette, subfamily G, member 2 (ABCG2),[49] and synuclein gamma,[50] which have been reported to be associated with resistance to chemotherapy and carcinoma development, respectively. This suggests that MSX2 also functions to enhance the biological aggressiveness of pancreatic cancer cells through pathways in addition to EMT, since many factors other than EMT contribute to the malignant phenotype of pancreatic cancer. On the other hand, the array analysis also revealed that the expression of snail, which is also a key regulator of EMT through reduced E-cadherin expression,[51] was higher in MSX2-expressing cells compared to controls. In addition, we recently revealed that MSX2 itself was indispensable for bone morphogenetic protein 4 (BMP4) induced EMT in pancreatic cancer cells.[52] In this context, MSX2 itself as well as many molecules or downstream pathways is likely to be involved in EMT in

pancreatic carcinoma cells. Therefore, in addition to Twist 1 or other molecules as stated above, we are investigating the MSX2-mediated molecules as candidate therapeutic targets in pancreatic cancer.

Acknowledgment

We thank Dr. C. Takahashi for vectors including MSX2 cDNA.

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Dig Dis Sci (2008) 53:2576–2582
DOI 10.1007/s10620-007-0154-1

ORIGINAL PAPER

The *PMAIP1* Gene on Chromosome 18 is a Candidate Tumor Suppressor Gene in Human Pancreatic Cancer

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Received: 4 September 2007 / Accepted: 26 November 2007 / Published online: 31 January 2008

Abstract Frequent loss of heterozygosity on the long arm of chromosome 18 is observed in pancreatic cancer. Previous studies suggested the existence of one or more tumor-suppressor genes other than *SMAD4* on chromosome 18. To identify the candidate tumor-suppressor gene(s), we compared gene expression by cDNA microarray analyses using a pancreatic cancer cell line Panc-1 and its hybrid cell lines showing suppressed cell growth after introduction of one normal copy of chromosome 18. The microarray analyses identified 38 genes on chromosome 18 that showed differential expressional levels. Among these genes, phorbol-12-myristate-13-acetate-induced protein 1 (*PMAIP1/APR/NOXA*) was identified as one of the candidates for tumor suppressor. Expression vector-mediated introduction of *PMAIP1* suppressed cell proliferation, and RNAi-mediated knockdown of *PMAIP1* induced recovery of cell growth. These results suggest that *PMAIP1* may play an important role in the progression of pancreatic cancer.

Keywords Pancreatic cancer · Tumor-suppressor gene · Chromosome 18 · *PMAIP1/APR/NOXA*

Introduction

It is well-known that pancreatic cancer is one of the leading causes of cancer deaths worldwide. Despite a variety of efforts aimed at improving the prognosis, its mean five-year survival rate still remains below 5% [1]. Although 10–20% of pancreatic cancer patients undergo surgery with curative intent, only one-fourth or less of these patients with successful operations will survive five years or more [2, 3]. There are two major problems:

1. the latency of the early symptoms and the lack of efficient detection methods prevent better prognosis and cause the low incidence of curative operations; and
2. pancreatic cancer itself harbors biological features that reduce the five-year survival rate to only one-fourth even after a curative operation.

To improve the prognosis for patients with pancreatic cancer, it is clearly necessary to obtain a better understanding of the molecular mechanisms of this deadly disease.

The results of molecular and pathological studies indicate that a variety of nonrandom genetic and genomic alterations are characteristic of pancreatic carcinomas [4]; gains of 5p, 7p, 8q, 17q, and 20q and losses of 1p, 3p, 9p,

Electronic supplementary material The online version of this article (doi:10.1007/s10620-007-0154-1) contains supplementary material, which is available to authorized users.

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12q, 17p, 18q, and 21q have been described by cytogenetic and allelotype studies [5–7], and *KRAS* [8], *TP53* [9], *CDKN2A* [10], and *SMAD4* [11] have been considered as major molecules that play key roles in tumorigenesis.

The loss of heterozygosity (LOH) of 18q at the locus for *SMAD4* is associated with poor patient prognosis and advanced tumor progression [12]. On the other hand, although frequent loss of 18q occurs in pancreatic pre-malignant lesions [13], expression of the SMAD4 protein is observed in all pancreatic intraductal papillary mucinous neoplasms, 50% of them showing 18q-LOH [14]. However, adenovirus-mediated transfer of *SMAD4* does not inhibit the growth of pancreatic ductal adenocarcinoma cell lines with completely inactivated *SMAD4* in vitro, but does inhibit the in-vivo growth by halting angiogenesis [15, 16]. Furthermore, microcell-mediated transfer of chromosome 18 can induce in-vitro and in-vivo growth suppression in pancreatic cancer cells, irrespective of SMAD4 activity [17]. Cytogenetic, allelotype, and somatic cell hybrid studies in human cancers other than pancreatic cancer suggest the possibility of a TSG(s) on 18q other than *SMAD4* that plays a role in the carcinogenesis of colorectal or prostate cancer [18, 19]. Accordingly, the available data suggest that:

1. two or more TSGs are present on 18q;
2. *SMAD4* is one of the TSGs on 18q, but it plays a role at a later stage of the disease; and
3. an unknown TSG(s) yet to be identified plays an important role at the early stage of carcinogenesis.

In this study, we compared the gene-expression profiles of pancreatic cancer cells before and after restoration of chromosome 18 with the aim of identifying TSG(s) on chromosome 18 other than *SMAD4*.

Materials and methods

Pancreatic cancer cell lines

The pancreatic cancer cell line used in this study was Panc-1, purchased from ATCC (Manassas, VA, USA), cultured according to the protocols of the suppliers using RPMI-1640 supplemented with 10% FBS, and well characterized mutationally [17, 20]; this cell line harbors three copies of chromosome 18cen with two copies of 18q, with wild type *SMAD4*. Two stable hybrid cells, Panc-1H(18)-1 and 2, each containing a normal copy of chromosome 18 and generated by the microcell-mediated chromosome transfer technique described previously [17], were used in this study. These cells were grown in RPMI-1640 supplemented with 10% FBS containing 400 μg/ml G418.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

Cell proliferation was monitored by an MTT assay, using methods described elsewhere [21], for one week in the absence of G418, and a proliferation index was calculated for each parental and corresponding hybrid cell line by methods described elsewhere [22]. In all assays, 1×10^3 cells of each cell type in 100 µl suspension were plated and incubated in flat-bottomed 96-well plates. The conversion of MTT to formazan dye with absorbance at 590 nm was spectrometrically measured using a multiwell plate reader. All experiments were performed in duplicate and repeated at least three times.

Tumorigenicity in the mice xenograft model

Tumorigenicity in the mice xenograft model was assayed using female six-week-old nude mice purchased from Clea Japan (Tokyo, Japan), in accordance with methods described elsewhere [23]. The mice were maintained under pathogen-free conditions and used in accordance with the guidelines of the NIH and the Tohoku University School of Medicine. Logarithmically growing cells trypsinized from subconfluent mono-layers were suspended in medium containing 25% Matrigel Growth Factor Reduced (Becton Dickinson Labware, Franklin Lakes, NJ, USA). For each inoculation, 1×10^6 cells in 0.2 ml suspension were injected s.c. into the hind flanks of nude mice. The tumor volume was estimated from the formula; $V = 0.4 \times D \times d^2$ (V = tumor volume, D = longitudinal diameter, and d = latitudinal diameter) at the time of weekly measurements.

Microarray analysis

Total RNAs were extracted from the cultured cells using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The oligonucleotide microarray analyses were then conducted using CodeLink UniSet Human 20 K I Bioarray (Amersham Biosciences, UK) according to the manufacturer's instructions. Processed slides were scanned using an Axon GenePix 4000 Scanner and GenePix Pro Software (Axon Instruments, Foster City, CA, USA). These data were analyzed using GeneSpring 6 software (Silicon Genetics, Redwood City, CA, USA) in accordance with methods described elsewhere [24].

Reverse transcription PCR (RT-PCR)

Total RNAs extracted from cell pellets were used for reverse transcription reactions with SuperScript III RNase

H^- reverse transcriptase (Invitrogen, San Diego, CA, USA) according to the manufacturer's protocol. Semiquantitative RT-PCR was performed in accordance with methods described elsewhere [25], and concentrations of template cDNAs were adjusted to give the same quantity by β-2 microglobulin (*B2M*) mRNA. Nucleotide sequences of the primers and optimized conditions for reactions are available on request to the authors.

Plasmid constructions and transfection

pcDNA6/myc-His A (pD6A) vectors (Invitrogen, Carlsbad, CA, USA) harboring the full length of the coding sequence of the *PMAIP1/APR/NOXA* gene at its *Hin*dIII/*Xho*I sites were prepared for expressing *PMAIP1* (pD6A-PMAIP1). The nucleotide sequence of PCR-amplified insert DNA was analyzed using an ABI PRIZM BigDye Terminator Cycle Sequencing FS Ready Reaction Kit and an ABI PRIZM 310 DNA Analyzer according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). For transfection, 1×10^4 cells were plated in 96-well plates and transfected with 0.2 μgof either pD6A or pD6A-PMAIP1 using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Short interference RNA transfection

Two sets of Duplexed Stealth siRNAs (Invitrogen) were used for the knockdown experiment of the *PMAIP1* gene. The siRNA sequences used against *PMAIP1* were as follows: siPMAIP1-1: UUUGUCUCCAAAUCUCCUGAGU UGA; siPMAIP1-2: AUCAGAUUCAGAAGUUUCUGCC GGA. A Stealth RNAi Negative Control Kit was used as the control. The siRNAs were dissolved in DEPC-treated water to a final concentration of 20 μmol/l. In-vitro transfection was done using the Oligofectamine reagent (Invitrogen) according to the manufacturer's instructions.

Results

In this study, we analyzed two independently established hybrid cells, Panc-1H(18)-1 and 2, after transfer of a normal copy of human chromosome 18 into Panc-1, one of the well characterized pancreatic cancer cell lines with wild type *SMAD4* [17]. We then re-estimated their in-vitro proliferation. The results, as outlined in Fig. 1a, showed that the in-vitro growth of one of the hybrid clones, Panc-1H(18)-2, showed significant suppression whereas growth of other clone, Panc-1H(18)-1, did not differ from that of parental cells.

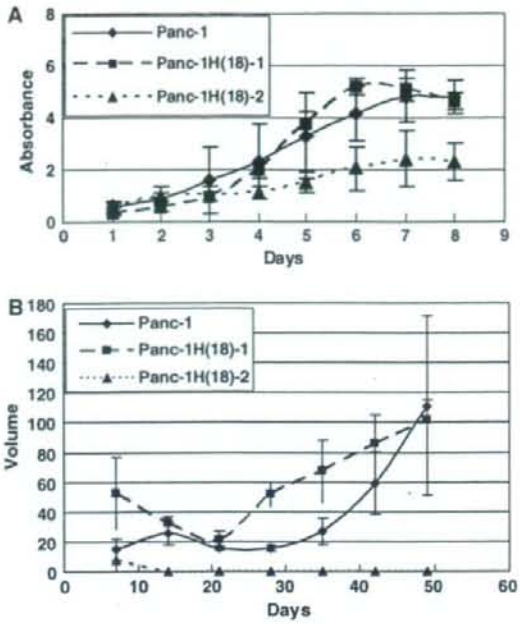

Fig. 1 (**a**) MTT assay monitoring the in-vitro proliferation. Parental Panc-1 and two hybrid cells, Panc-1H(18)-1 and 2, were seeded on 96-well plates, and absorbance of MTT was measured every day. The growth rate of Panc-1H(18)-2 was significantly slower than that of its parental Panc-1 cell or the other hybrid, Panc-1H(18)-1. (**b**) In-vivo tumorigenic assay. A total of 1×10^6 cells of the parental Panc-1 and two hybrid clones, Panc-1H(18)-1 and 2, were inoculated subcutaneously into nude mice, and the tumor volumes were measured every week. The growth rate of Panc-1H(18)-2 was significantly suppressed compared with the parental Panc-1 and the other hybrid clone, Panc-1H(18)-1

We then examined the in-vivo tumorigenic phenotypes of the hybrid cells by inoculating them into three nude mice and comparing them with parental cells. In order to shorten tumor latency and enhance tumor growth, we mixed the cells in a suspension containing Matrigel extract. As shown in Fig. 1b, a hybrid cell line Panc-1H(18)-2 showed a significant reduction in tumor volume and a longer latency when compared with the parental cell, Panc-1, and the other hybrid cell line, Panc-1H(18)-1.

These results indicated that one of the hybrid cell lines, Panc-1H(18)-2, had significant reductions in both in-vitro proliferation and in-vivo tumorigenic activity. We regarded this clone as a model of pancreatic cancer in which chromosome 18 has been restored. The other clone, Panc-1H(18)-1 was used as the control in this experiment. Results of the microsatellite analyses showed a large deletion; in our analysis, we found no differences from results obtained with the parental cell line, Panc-1 (data not shown). Thus, there are two possible explanations:

1. Panc-1H(18)-1 has deleted the gene responsible for the suppression of growth in vitro and in vivo during the successive cultures after the initial chromosome transfer; and
2. this cell line was a mixture of two or more cells including the parental Panc-1 cell as a minor population at the moment of establishment.

In the latter case, parental Panc-1 cells grew faster than the hybrid cells and finally prevailed in the cell-culture dishes.

To determine the differences in gene-expression profiles between the parental Panc-1 and its hybrid clones we performed cDNA microarray analyses. The results gave us information about the genes that were up-regulated on chromosome 18. After comparing the expression profiles of Panc-1H(18)-2 to the parental Panc-1 and Panc-1H(18)-1 we selected genes on chromosome 18 with differential values of more than 1.5-fold to the parental cells and 2-fold to Panc-1H(18)-1 clones to make a list of genes of adequate number (see supplementary material, Table 1). We selected 38 such genes, and they could account for the differences in tumorigenic phenotypes between Panc-1H(18)-2 clone and the others (the parental Panc-1 and the hybrid Panc-1H(18)-1). Predicted functions were annotated on the basis of the Gene Ontology database. This comparison may give us significant information about genes accounting for the tumor-suppressive phenotype without noise from the MMCT technique itself.

We validated the results of the microarray experiment by the semiquantitative RT-PCR method (Fig. 2), and the results were not completely consistent with the corresponding data from the microarray experiments in the magnitude of change in expression level. Among these genes, we selected *PMAIP1*, synonym for *NOXA*, as a candidate TSG on chromosome 18 for further analysis, because this gene up-regulated only in Panc-1H(18)-2 among these three cells.

We next investigated the effects of overexpression of *PMAIP1* on the in-vitro growth of the two hybrid cells. We compared the effect by introduction of both the full-length *PMAIP1* coding sequence and the empty pD6A vector; cellular proliferation of the parent cells and the hybrids was monitored by MTT assay for one week. As shown in Fig. 3, cell proliferation was significantly suppressed by introduction of *PMAIP1* compared with mock vector in the cell lines analyzed, but stronger suppression was observed in Panc-1H(18)-1 than Panc-1H(18)-2.

To verify that *PMAIP1* is involved in cell growth, siRNA experiments were done on Panc-1H(18)-2. The ability of *PMAIP1* siRNA to suppress *PMAIP1* expression was confirmed by semiquantitative RT-PCR (Fig. 4a). The effect of knockdown of *PMAIP1* on tumor growth was then measured by MTT assay. Panc-1H(18)-2 cells treated with *PMAIP1* siRNA showed increased growth, although not to a significant level, compared with cells treated with control siRNA (Fig. 4b). These results imply partial involvement of *PMAIP1* in the tumor-suppressive pathway of pancreatic carcinogenesis.

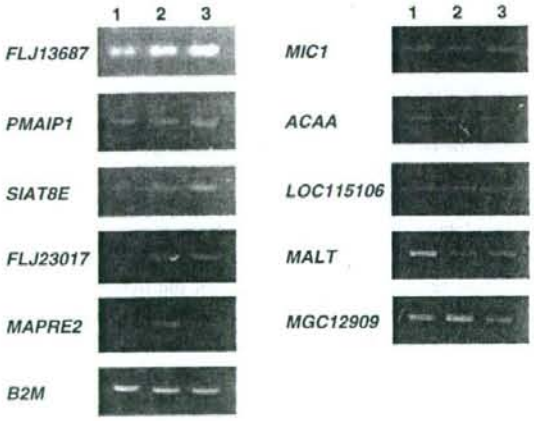

Fig. 2 Results from semiquantitative RT-PCR of ten genes extracted from microarray analyses are shown. β-2 microglobulin (*B2M*) mRNA was used as the internal control. Results were observed by agarose gel electrophoresis followed by ethidium bromide staining. *Lane 1*, Panc-1; *lane 2*, Panc-1H(18)-1; *lane 3*, Panc-1H(18)-2

Discussion

Several lines of evidence have suggested that chromosome 18q may carry a TSG(s) other than *SMAD4* that plays a

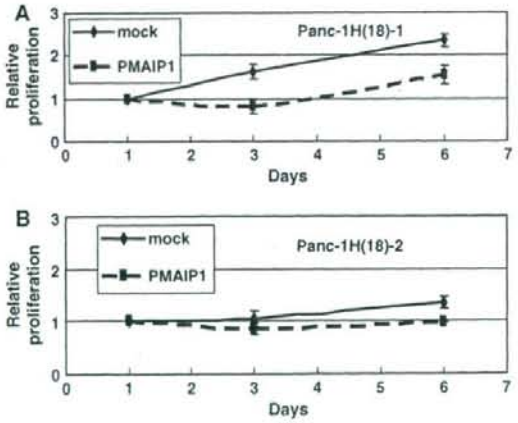

Fig. 3 *PMAIP1* was introduced into the two cell lines Panc-1H(18)-1 (**a**), and Panc-1H(18)-2 (**b**) by expression vector pD6A-PMAIP1. The empty vector also used is indicated by *mock*. Note that significant growth suppression was observed in each cell line

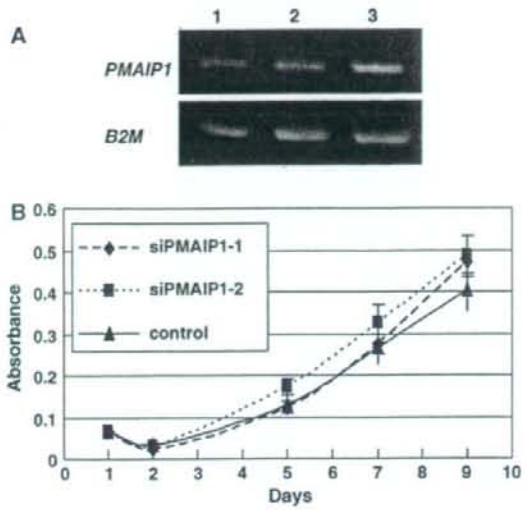

Fig. 4 RNAi-mediated knockdown of *PMAIP1*. (a) Two sets of siRNA for *PMAIP1*, siPMAIP1-1 and 2, were transfected into Panc-1H(18)-2, and down-regulation of *PMAIP1* was observed by RT-PCR. Both siRNAs for *PMAIP1* suppressed the expression level of *PMAIP1* compared with cells transfected with control siRNA, using a Stealth RNAi Negative Control Kit. *Lane 1*, siPMAIP1-1; *lane 2*, siPMAIP1-2; and *lane 3*, control siRNA. (b) Effect of siRNA-mediated knockdown against *PMAIP1* on Panc-1H(18)-2. The siRNAs for *PMAIP1*, siPMAIP1-1 and 2, and Stealth RNAi Negative Control Kit as the control, were transfected in Panc-1H(18)-2, and cell proliferation was monitored by MTT assay. The cells treated with siRNA showed a slight recovery of proliferation compared with the cells treated with control siRNA

role(s) in the development and/or progression of pancreatic cancer [4]. In the current study we aimed to gather functional evidence for the existence of an unknown TSG(s) and refine candidate(s) yet to be identified on the 18q arm. MMCT has been proved to be a useful tool for providing functional evidence for the identification of a TSG(s) in a variety of cancers, for example colon cancer [18, 26], prostate cancer [19], and melanoma [27]. This technique also led the way to isolation of the *NBS* gene [28].

One of the derived hybrids, Panc-1H(18)-2, showed significant suppression of proliferation and tumorigenesis compared with both the parental Panc-1 and the other hybrid Panc-1H(18)-1 cells. These results suggest that the newly introduced chromosome 18 in Panc-1H(18)-2 harbors a TSG(s) that can overcome defective functions of existing genes in Panc-1.

Microarray analysis using a total of 20,000 unique human genes successfully delineated the gene-expression profiles of Panc-1H(18)-2 in comparison to parental cells and the other hybrid, Panc-1H(18)-1. Microarray analysis can only detect the genes in which expression levels alter. If the major mechanisms of inactivation of the TSG(s) on chromosome 18 are structural changes such as nonsense or frameshift mutations, we cannot get to the responsible gene(s) by microarray analysis. Furthermore, we would have been able to examine only about two-thirds of the total human genes in this study. Nonetheless, we still think that microarray analysis using cells before and after MMCT is one good strategy that we should employ in order to explore the genes that play important roles in pancreatic carcinogenesis; it was successfully utilized on chromosome 12 previously [25].

A total of 38 genes on chromosome 18 were selected as candidate TSGs; these genes are predicted to function in a variety of pathways and situations, potentially indicating complicated molecular networks underlying cellular phenotypes triggered by genes on the transferred chromosome and/or the effect of introduction of one additional allele itself. Among those selected genes, several interesting genes have been reported in association with apoptosis. In this study, we identified *PMAIP1*, also named *APR* or *NOXA*, as a gene strongly associated with in-vitro and in-vivo proliferation and frequently down-regulated in pancreatic cancer. PMAIP1, a member of the BH3-only subfamily of Bcl-2 family proteins, heterodimerizes and antagonizes the activity of prosurvival proteins such as Bcl-2 and Bcl-xl, thus promoting apoptosis [29, 30]. PMAIP1 is induced by p53 [29, 31, 32], HIF-1α [33], interferon [34, 35], or anti-cancer drugs [36–39]. Vector-mediated expression of the *PMAIP1* gene suppressed proliferation of the cells, and RNAi-mediated knockdown of *PMAIP1* in Panc-1H(18)-2 showed a tendency toward growth stimulation when compared with the cells treated with control siRNA; *PMAIP1* could be one of the candidate TSGs on chromosome 18 that is involved in pancreatic ductal carcinogenesis.

In conclusion, our results support the idea of the existence of TSGs on chromosome arm 18q, and *PMAIP1* is one of the candidates. However, the growth-suppressive ability of introduction of *PMAIP1* was not as effective as that of chromosome transfer. Furthermore, microarray analysis cannot detect structural alterations that are one of the frequent causes for human carcinogenesis, as we discussed above. In addition, it is not easy to identify small differences in expression by microarray analysis, even if they are important for tumorigenesis. There is a possibility of existence of other TSG(s) on 18q, and a group of genes on 18q, including *PMAIP1*, that plays a role as a tumor suppressor. Because of these limitations of microarray analysis, the development of a better algorithm for analysis is necessary. Recently-discovered miRNAs are also candidates for the gene(s) responsible for carcinogenesis that need to be elucidated.

Acknowledgments We are grateful to Dr B.L.S. Pierce (University of Maryland University College) for editorial work in the preparation

of this manuscript. This work was supported in part by Grants-in-Aid and the 21st Century COE Program Special Research Grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by a Grant-in-Aid for Cancer Research from the Ministry of Health, Labour and Welfare of Japan.

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The American Journal of Surgery (2008) 196, 125–129

The American Journal of Surgery®

How I Do It

Extensive hilar bile duct resection using a transhepatic approach for patients with hepatic hilar bile duct diseases

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KEYWORDS:
Benign bile duct stricture;
Bile duct resection;
Hilar cholangiocarcinoma;
Transhepatic approach

Abstract

BACKGROUND: Extensive hilar bile duct resection beyond the second- or third-order intrahepatic biliary radicals is usually required for patients with hilar cholangiocarcinoma as well as those with benign inflammatory stricture. Most hilar cholangiocarcinoma is resected with combined major hepatectomy to obtain tree surgical margins. The purpose of this study was to show the surgical procedure and the usefulness of extensive hilar bile duct resection using a transhepatic approach for patients with hilar bile duct diseases.

METHODS: Five patients with hepatic hilar bile duct disease and who were unfit for major hepatectomy for several reasons underwent extensive hilar bile duct resection by way of a transhepatic approach. Four of the patients had hilar bile duct cancer, including 1 with mucous-producing bile duct cancer of low-grade malignancy and 1 with a postsurgical benign bile duct stricture.

RESULTS: After extensive hilar bile duct resection, bile duct stumps ranged in number from 3 to 7 mm (mean 3.4). Surgical margins of bile duct stump were free of cancer in all 5 cancer patients. The long-term outcomes were as follows: 3 patients are alive at the time of publication, and 2 patients have died.

CONCLUSIONS: A transhepatic approach may be useful when performing extensive hilar bile duct resection bile duct stricture of biliary disease at the hepatic hilus, especially in high-risk patients who are unfit for major hepatectomy as well as in those having benign bile duct stricture and low-grade malignancy.

Most biliary tract cancers involving the hilar bile duct, such as hilar cholangiocarcinoma and gallbladder carcinoma, must be resected by hepatectomy and extrahepatic bile duct resection to obtain cancer-free surgical margins. However, in patients who are unfit for major hepatectomy because of liver dysfunction and general poor condition, combined major hepatectomy with bile duct resection might incur increased surgical risk.[1–3] In contrast, low-grade malignancies, such as mucin-producing bile duct tumors, may sometimes be radically resected without performing combined major hepatectomy, even when there is a tumor in the upper third of the extrahepatic bile duct.[4,5] We applied a transhepatic approach to extensive hilar bile duct resection in 5

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Manuscript received February 21, 2007; revised manuscript April 19, 2007

0002-9610/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.amjsurg.2007.04.020

Table 1 Patient characteristics and operative data

Patient no/age (y)	Sex	Disease	No. of BDS	Surgical data Time	Blood loss (mL)
1/61	Male	Bile duct cancer	7	7 h 16 min	390
2/76	Male	Benign stricture	4	6 h 38 min	1,023
3/76	Male	Bile duct cancer	3	5 h 32 min	465
4/80	Male	Bile duct cancer	3	7 h 18 min	680
5/72	Male	Bile duct cancer	5	8 h 13 min	1,400

BDS = bile duct stumps.

patients with hilar bile duct disease. This approach may provide a sufficient surgical view and facilitate extensive resection of the hilar bile duct, facilitating reconstruction by bilioenteric anastomosis. The purpose of this study was to show the usefulness of extensive hilar bile duct resection using a transhepatic approach for patients with hilar bile duct diseases.

Methods

Between October 2001 and December 2006, 5 patients with hepatic hilar bile duct disease underwent extensive hilar bile duct resection using a transhepatic approach. The patients' characteristics are listed in Table 1. There were 4 patients with hilar bile duct cancer, including 1 with mucous-producing bile duct cancer of low-grade malignancy, and 1 with postsurgical benign bile duct stricture. The ages of the 5 patients ranged from 61 to 80 years. Selection criteria for this surgical procedure, used in the 4 patients with bile duct cancer, were that the cancer was localized at the hilar bile duct had not invaded the extramural liver or vessels, such as the portal vein and the hepatic artery, and that curative resection with extensive hilar bile duct resection alone was indicated for treating the cancer based on the evaluation of preoperative imaging findings. Furthermore, the surgical stress of major hepatectomy was deemed to be unacceptably extreme for 3 patients because of advanced age, liver dysfunction, or otherwise general poor condition (Table 2).

Surgical procedures

Under a subcostal transverse skin incision in the upper abdomen, the right hepatic lobe was mobilized by dissecting the coronary ligament. The gallbladder was mobilized from the gallbladder bed of the liver. The hepatoduodenal ligament was skeletonized, and the portal vein and the hepatic artery were tracked down into the intrahepatic Glissonian sheath until the second-order intrahepatic Glissonian sheath at the right side and the umbilical portion at the left side. The liver parenchyma was transected along the Cantlie line as the interlobar border between the right and the left hepatic lobes after identifying the demarcation line by hemilobar inflow vascular clamping.

By identifying the hepatic vein branch from the right anterior segment (S5 and S8) and the left medial segment (S4), we determined on which side of the middle hepatic vein the transected line would be situated. Preoperative imaging, especially enhanced computed axial tomography (CAT), is important for identification of the branch of the major hepatic vein before applying the transhepatic approach. In addition, intraoperative Doppler ultrasonography could be useful for identifying the branches of the major hepatic vein. If the vein branch from segments 5 or 8, with a confluence into the middle hepatic vein, had a larger caliber than the vein branch from segments 5 or 8 with a confluence into the right hepatic vein, it was judged to be significant drainage vein that should be preserved. Similar judgment was rendered in the vein branch from segment 4 with a confluence into the middle or left hepatic veins. Therefore, in the case of remarkable hepatic vein branches of the right anterior segment with a confluence into the middle hepatic vein, the liver parenchyma was transected along the left side of the middle hepatic vein without ligating these vein branches to avoid congestive injury of the right anterior hepatic segment (Fig. 1). In contrast, in the case of significant hepatic vein branches of the left medial segment with a confluence into the middle hepatic vein, the liver should be transected on the line along the right side of the middle hepatic vein (Fig. 2), or the these veins should be preserved as much as possible by not transecting the liver

Table 2 Reasons for avoiding major hepatectomy in 5 patients who underwent surgical resection by way of transhepatic approach

Patient no	Reasons
1	Localized bile duct tumor of low-grade malignancy
2	Benign stricture, old age, liver dysfunction (ICG 20%)
3	Localized bile duct cancer, old age, liver dysfunction (ICG 15%)
4	Old age, liver dysfunction (ICG 15%)
5	Localized bile duct cancer, liver dysfunction (ICG 14%)

ICG = indocyanine green; retention rate at 15 minutes after the intravenous injection of 0.5 mg/kg body weight.

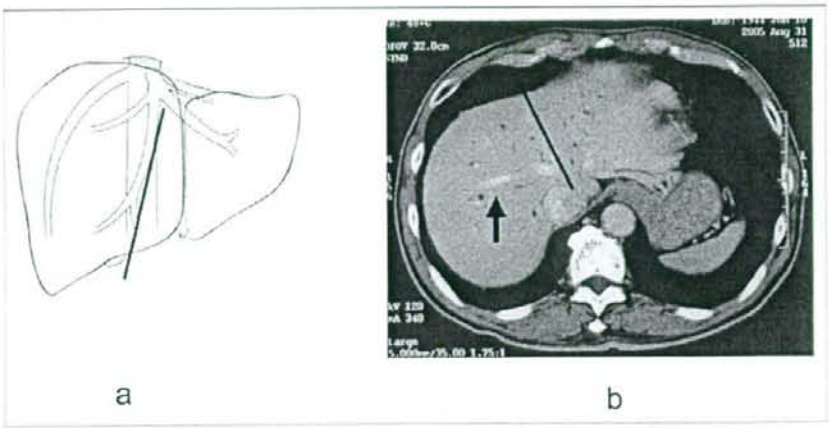

Figure 1 A large drainage vein from segment 8 pouring into the middle hepatic vein in illustration (1a) and on CAT (1b).

completely on the line along the left side of the middle hepatic vein.

After transecting the liver parenchyma between the right and the left lobes by CUSA under inflow vascular clamp, it is possible to expose the hepatic hilar bile duct extensively without excising any liver volume (Fig. 3). The lower bile duct should be ligated and divided at the level of the intrapancreatic portion as low as possible to obtain a negative margin of the bile duct stump. It is possible to resect the upper bile duct at the level of the second-or third-order branch of the intrahepatic bile duct bilaterally (Fig. 4). Therefore, at the right side of the hepatic transected plane, there will be several bile duct stumps of the anterior and posterior segments. At the left side of hepatic transected plane, there will usually be 2 or 3 bile duct stumps, such as B2 from segment 2, B3 from segment 3 of the lateral segment, and B4 from the medial segment of the liver. After extensive resection of the hilar bile duct and extrahepatic bile duct, bilioenteric anastomosis can easily be performed with a single-layer interrupted suture of 5-0 PDSII (Ethicon) under sufficient surgical view in an end-to-side fashion by Roux-en-Y loop of the jejunum. Biliary stent tubes should routinely be placed in each anastomosis through the retrograde transhepatic route (Fig. 5). However, with bile duct stump diameter <2 mm, a thin biliary stent tube should be placed through the transjejunum route. Hemostasis on the liver transected surface should be carefully performed before the abdomen is closed.

Results

The surgery data of 5 patients are listed in Table 1. After extensive hilar bile duct resection, bile duct stumps ranged in size from 3 to 7 mm (mean 4.4). Surgical margins at bile

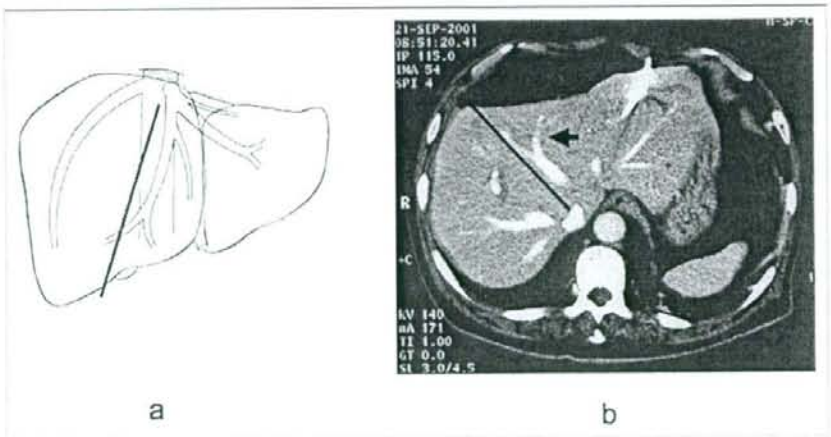

Figure 2 A large drainage vein from segment 4 pouring into the middle hepatic vein in illustration (2a) and on CAT (2b).

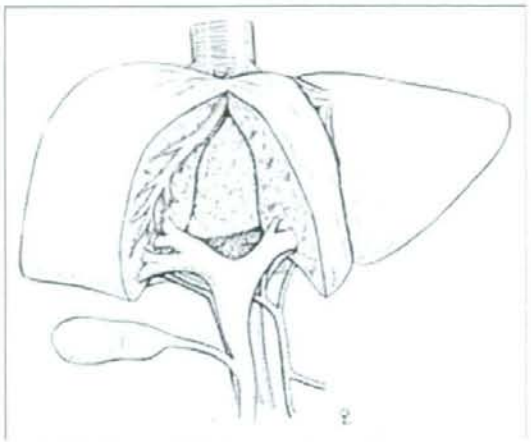

Figure 3 Sufficient, extensive exposure of the hilar bile duct after liver transection.

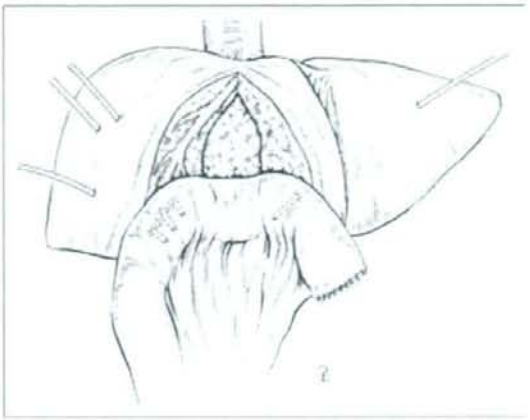

Figure 5 Bilioenteric anastomosis, with sufficient surgical view, using a Roux-en-Y jejunal loop.

duct stumps were free of cancer in all 4 patients with hilar bile duct cancer. Surgery time ranged from 5 hours 32 minutes to 8 hours 13 minutes (mean 7 hours), and surgical blood loss ranged from 390 to 1400 g (mean 792). There were no surgical deaths, but 1 patient suffered from biliary fistula that required a lengthy hospital stay because of delayed healing. Hospital stays ranged from 28 to 97 days (mean 47). The postsurgical outcomes were as follows: 3 patients (2 with bile duct cancer and 1 with benign bile duct stricture) are alive (during early observation periods of 8 to 38 months) at the time of publication, and 2 cancer patients have died (Table 3).

Comments

The transhepatic anterior approach was recently reported by Yamamoto et al[6] as useful in surgical resection for patients with liver cancer present in the caudate lobe and also by Chi-Leung et al[7] for resection in patients with hugeliver cancer. They also reported the usefulness of a transhepatic anterior approach in patients with liver cancer, especially in those who had undergone isolated caudate lobectomy for deeply localized liver cancer, and in patients with right hemihepatectomy for huge liver cancers, such as those invading the retroperitonium and diaphragma. Herein we propose the usefulness of a transhepatic approach for patients with bile duct disease at the hepatic hilus, such as benign hilar bile duct stricture, and patients with low-grade malignant or localized hilar cholangiocarcinoma, especially those who are unfit for major liver resection.

This approach can offer a sufficient surgical view to enable clear and extensive visualization of the hilar bile duct, including the second- and third-order branches of the intrahepatic bile duct. This may enable extensive hilar bile duct resection without excision of any volume of liver parenchyma, which may be appropriate for patients with liver dysfunction and for those in whom surgical major hepatectomy is contraindicated. Templeton and Dodd[8] and Waddell[9] previously reported the similar approach of anatomic separation of the

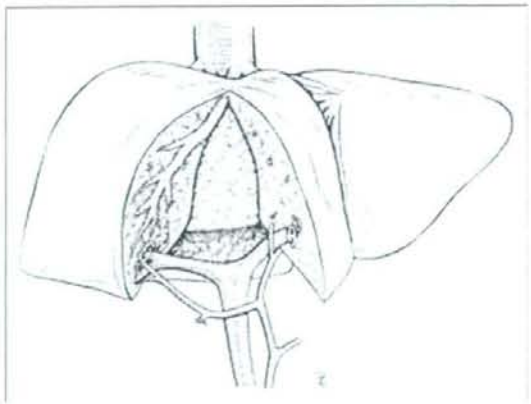

Figure 4 Numerous intrahepatic bile duct stumps are clearly exposed after extensive hilar bile duct resection.

Table 3 Outcomes of 5 patients who underwent surgical resection by way of transhepatic approach

Patient no	Complications	Hospital stay (d)	Long-term outcome
1	—	28	Alive at 30 months
2	—	53	Alive at 28 months
3	Biliary fistula, pleural effusion	97	Alive at 23 months
4	—	28	Died at 38 months
5	Wound infection	29	Died at 29 months

right and left lobes of the liver to expose intrahepatic bile ducts in the patients with upper bile duct stricture. However, they did not describe the liver transecting line in detail, especially concerning the point of hepatic venous congestion. We previously reported refined approaches to central hepatectomy for hilar cholangiocarcinoma, such as segment 1 and 4 hepatectomy, as parenchyma-preserving types of hepatectomy[10,11] and have shown their usefulness for patients for whom major hepatectomy would incur high risk. It has been reported in many previous articles that the caudate lobe should be excised in most patients with hilar cholangiocarcinoma because cancer invasion extends into the intrahepatic bile duct branch of segment 1.[12,13] Therefore, extensive hilar bile duct resection must be used only in patients with hilar cholangiocarcinoma in whom cancer invasion seems not to extend into the intrahepatic bile duct branches of segment 1. The correct evaluation of these findings may require meticulous preoperative imaging findings and may sometimes be difficult. In contrast, hilar bile duct tumor of low-grade malignancy may not invade deeply into the intrahepatic bile duct branch of segment 1.[4,5] In our patient (patient no. 1) with mucous-producing bile duct tumor, pathologic findings showed no clear invasion into the intrahepatic bile duct branch of segment 1.

The second advantage of this procedure is that it provides a sufficient surgical view after extensive hilar bile duct resection, thus facilitating recognition. Using the standard procedure, ie, hilar bile duct resection without liver parenchymal transection, it is usually difficult to obtain sufficient favorable surgical view for segment I resection when the surgeon is reconstructing the bilioenteric bypass. We did not encounter failure of bilioenteric anastomosis in any of the 5 patients in our series. In 1 patient (patient no. 3), biliary fistula appeared and persisted for a long time after surgery. However, in this patient, biliary fistula was induced by bile leakage from the transected liver surface, not from leakage of the bilioenteric anastomosis. The broad transected surface of the liver may be a disadvantage of the transhepatic approach. However, surgical blood loss was not such that blood transfusion was required in any patient in our series.

In the transhepatic approach, the liver parenchyma should be transected the length of the boundary between the right and left hepatic lobes, along which the middle hepatic vein runs. Therefore, a decision must be made as to along which side of the middle hepatic vein should the liver parenchyma be transected: the right side or the left side of the wall? When considering which to choose, it is important to recognize the segmental drainage veins from segments 4 and 8 into the middle hepatic vein. If the segmental drainage vein from segment 4 makes a confluence into the left hepatic vein, the transhepatic approach may be followed along the left side of the middle hepatic vein (Fig. 2). The segmental drainage vein from segment 8 is sometimes a major drainage vein from the anterior segment, with a confluence into the middle hepatic vein.[14,15] In such patients, right-sided transection should be avoided as much as possible to lessen the chance of postsurgical congestion and subsequent damage to the anterior segment of the liver (Fig. 1). Therefore, it is important to evaluate the drainage veins of segments 4 and 8 before and after surgery when the transhepatic anterior approach is used. In fact, there was no serious postsurgical liver dysfunction in any of the 5 patients of our series.

In conclusion, the transhepatic approach may be useful when performing extensive hilar bile duct resection in patients with biliary disease at the hepatic hilus, especially in high-risk patients unfit for major hepatectomy, as well as in the patients with benign bile duct strictures and low-grade malignancies.

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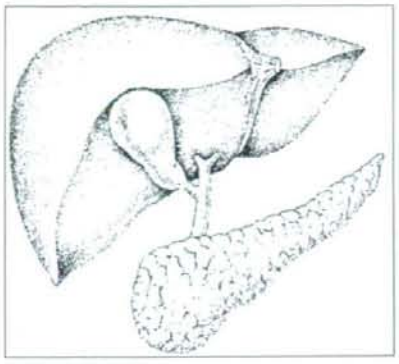

Hepato-Biliary-Pancreatic Surgery

Springer

Volume 15 Number 1 2008

J Hepatobiliary Pancreat Surg (2008) 15:15–24
DOI 10.1007/s00534-007-1276-8

Journal of HBP Surgery

Risk factors for biliary tract and ampullary carcinomas and prophylactic surgery for these factors

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Abstract
Curative resection is the only treatment for biliary tract cancer that achieves long-term survival. However, patients with advanced biliary tract cancer have only a limited prognosis even after radical surgical resection. Thus, to improve the long-term results, the early detection of biliary tract cancer and subsequent cure seem to be essential. The purpose of this study was to review the literature concerning the risk factors for cancerous and precancerous lesions of the biliary tract, and prophylactic surgery for these factors. It has been reported that pancreaticobiliary maljunction (PBM) with bile duct dilatation is a risk factor for gallbladder cancer and bile duct cancer, while PBM without bile duct dilatation is a risk factor for gallbladder cancer. Thus, in the former group, a prophylactic excision of the common bile duct and gallbladder should be recommended, while in the later group, a prophylactic cholecystectomy without bile duct resection may be the appropriate surgical procedure. It has also been reported that primary sclerosing cholangitis (PSC) is a risk factor for cholangiocarcinoma. Patients with PSC often develop advanced cholangiocarcinoma with a poor prognosis. In patients with PSC, therefore, strict follow-up should be recommended. Adenoma and dysplasia have been regarded as precancerous lesions of gallbladder cancer. A polypoid lesion of the gallbladder that is sessile, has a diameter greater than 10 mm, and /or grows rapidly, is highly likely to be cancerous and should be resected. Although gallstones seem to be closely associated with gallbladder cancer, there is no evidence of a direct causal relationship between gallstones and gallbladder cancer. Thus, a cholecystectomy is not advised for asymptomatic cholecystolithiasis. Controversy remains as to whether adenomyomatosis of the gallbladder and porcelain gallbladder are associated with gallbladder cancer. With respect to ampullary carcinoma, adenoma of the ampulla is considered to be a precancerous lesion. This article discusses the risk factors for cancerous and precancerous lesions of the biliary tract and prophylactic treatment for these factors.

Key words Biliary tract neoplasms · Risk factors · Prophylaxis therapy · Gallstones · Pancreaticobiliary maljunction · Precancerous conditions · Gallbladder · Guidelines

Offprint requests to: M. Miyazaki
Received: October 1, 2007 / Accepted: October 22, 2007

Introduction

One of the possible causes of biliary tract cancer may be chronic and continuous stimulation of the biliary tract, and cholangitis due to gallstones and the reflux of pancreatic juice into the biliary tract.[1,2] The underlying diseases that potentially cause such a condition include pancreaticobiliary maljunction (PBM),[3] primary sclerosing cholangitis (PSC),[4–7] chronic cholecystitis,[1] gallstones,[1,8] and adenomyomatosis.[9] In such patients, chronic inflammation potentially causes pathological changes of the biliary epithelium resulting in precursors of biliary tract cancer.[1]

Although curative resection is the only treatment for biliary tract cancer that achieves long-term survival, patients with advanced cancer have only a limited prognosis even after radical surgical resection. To improve the long-term results, therefore, the early detection of precancerous and cancerous lesions, and subsequent cure seem to be essential.

In this article, we discuss the predisposing factors for bile duct cancer, gallbladder cancer, and ampullary car-

cinoma, using a format of clinical questions (CQs) and responses. In the responses to the CQs, recommendations for treatment are noted (grades of these recommendations are defined in Table 1[10]). Also, levels of evidence are given (in parentheses) for findings in reference citations (see definitions of levels in Table 2[10]).

CQ 1 What are the risk factors for biliary tract and ampullary carcinomas?

Pancreaticobiliary maljunction (PBM) with bile duct dilatation and primary sclerosing cholangitis (PSC) are risk factors for biliary tract cancer (see side memos 1 and 2). PBM, particularly that without bile duct dilatation, is a risk factor for gallbladder cancer.

There are no evident risk factors for ampullary carcinoma.

The prevalence rate of biliary tract cancer varies in different geographical regions. Chile and Japan have the highest rate in the world, followed by East Asia and India (level V).[1,2] Also, there are age differences in the incidence of biliary tract cancer.[1] It has been reported that the risk factors for biliary tract cancer are, possibly, chronic and continuous stimulation and inflammation of the biliary tract.[1,2] Polypoid lesions of the gallbladder and adenomyomatosis have been regarded as a risk factors for gallbladder cancer. In this section, we review the respective risk factors for bile duct cancer, gallbladder cancer, and ampullary carcinoma.

Table 1. Strength of recommendations[10]

A, Strongly recommend performing the clinical action
B, Recommend performing the clinical action
C1, The clinical action may be considered although there is a lack of high-level scientific evidence for its use. May be useful
C2, Clinical action not definitively recommended because of insufficient scientific evidence. Evidence insufficient to support or deny usefulness
D, Recommend not performing the clinical action

Table 2. Levels of evidence[10]

Level I	Systematic review/meta-analysis
Level II	One or more randomized clinical trials
Level III	Nonrandomized controlled trials
Level IV	Analytic epidemiology (cohort studies and case-control studies)
Level V	Descriptive study (case reports and case-series studies)
Level VI	Opinions of expert panels and individual experts not based on patient's data

Risk factors for bile duct cancer

Pancreaticobiliary maljunction (see CQ 2)

A retrospective nationwide survey (1990 to 1999) of PBM in Japan revealed that 10.6% of PBM patients with bile duct dilatation were complicated by biliary tract cancer, and 33.6% of these biliary tract cancers were bile duct cancer[3] (level IV). PBM with bile duct dilatation is considered as a risk factor for bile duct cancer.

Primary sclerosing cholangitis (PSC)

Patients wit PSC carry an increased risk of bile duct cancer. Five percent to 10% of PSC patients develop bile duct cancer[4–7] (level V). Bile duct cancer associated with PSC is often advanced with a poor prognosis. PSC, therefore, should be recognized as a risk factor for bile duct cancer.

Controversy remains as to whether bile duct cancer is related to chronic inflammation due to gallstones or some gene mutations[8,11,12] (level IV).

Risk factors for gallbladder cancer

Pancreaticobiliary maljunction (see CQ 2)

There have been many studies that reported PBM as a risk factor for gallbladder cancer. A nationwide survey (1990 to 1999) of PBM revealed that the prevalence rate of biliary tract cancer was 10.6% in the group with bile duct dilatation, while the prevalence rate was 37.9% in the group without bile duct dilatation[3] (level IV). With respect to the PBM patients who developed biliary tract cancer, the incidence of gallbladder cancer was 64.9% in those with PBM with bile duct dilatation, whereas the incidence was 93.2% in those with PBM without bile duct dilatation. Therefore, PBM is an evident risk factor for gallbladder cancer. Of note, the frequency of gallstones in patients with PBM associated with gallbladder cancer is low.[13]

Gallstones and porcelain gallbladder (see CQ 3)

It has been well established that gallstones are closely associated with gallbladder cancer[1,8] (level V). It has also been reported that a stone size of more than 3 cm,

a family history of gallbladder cancer, and the duration of cholelithiasis are potential risk factors for developing gallbladder cancer.[1,14-16] However, there is no evidence of a direct causal relationship between gallstones and gallbladder cancer. Gracie and Ransohoff[17] followed-up the subsequent history of 123 patients with asymptomatic gallstones for 10 years or longer, and revealed that there was no case of gallbladder cancer reported among that group.

Controversy remains as to whether patients with "porcelain gallbladder" carry a risk of gallbladder cancer. It has been reported that porcelain gallbladder is often complicated by gallbladder carcinoma[18,19] (level IV), while another report suggests that porcelain gallbladder is not associated with gallbladder carcinoma[20] (level IV).

Adenoma of the gallbladder (Figs. 1 and 2; also see CQ 4)

There is consensus regarding the existence of two models through which malignant transformation is produced: the adenoma-carcinoma sequence and the dysplasia-carcinoma sequence. Intestinal and gastric metaplasias appear to be the pathway through which epithelial dysplasia is produced.[21] Yamagiwa[22] examined 110 cases of resected gallbladder carcinoma and found dysplasia adjacent to carcinoma in 46 of the 110 cases, and this change was frequently found in lesions at an early stage and in well-differentiated carcinoma (level V).

Kubota et al.[23] reported that in patients with polypoid lesions of the gallbladder, the respective diameters of adenomas and cancers were 6.9 mm (range, 4 to 13 mm) and 25.7 mm (range, 5 to 50 mm). In that study, 75% of the adenomas and 13% of the cancers had a diameter of less than 10 mm. It has also been reported that when a polypoid lesion of the gallbladder is sessile, has a diameter greater than 10 mm, and /or grows rapidly, it is highly likely to be cancerous.[23-25] In such cases, surgical resection should be recommended.

Adenomyomatosis (Fig. 3; 4)

Adenomyomatosis has not been considered to have malignant potential.[1] Nabatame et al.[9] studied the relationship between adenomyomatosis and gallbladder cancer by examining 4560 gallbladders (2031 from male patients and 2529 from female patients; age 14 to 94 years) resected for gallbladder cancer, gallstones, or other diseases. In that study, the incidence of gallbladder carcinoma was higher in patients with segmental adenomyomatosis (22/334; 6.6%) than in those without (181/4226; 4.3%; $P = 0.049$). This difference was more marked in patients equal to or older than 60 years of age ($P < 0.001$). However, the magnitude of risk for gallbladder cancer in patients with adenomyomatosis has not been clearly established.[1,26]

Risk factors for ampullary carcinoma

Kimura et al.[27] histologically investigated the papilla of Vater in 576 autopsy cases of elderly people and revealed that the incidences of group 3 and 4 epithelia in the common channel were significantly higher than those in the intraduodenal portion of the bile duct, pancreatic duct, or duodenal epithelia. They also investigated

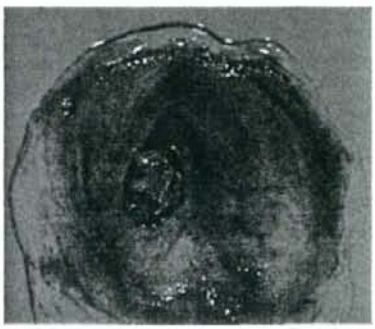
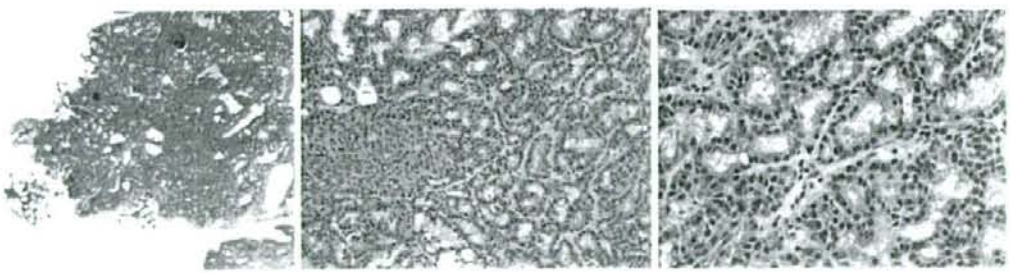


Fig. 1. Macroscopic photograph of adenoma of the gallbladder



a b,c

Fig. 2a–c. Histological examination of adenoma of the gallbladder. **a** Low magnification; **b** intermediate magnification; **c** high magnification (H&E)

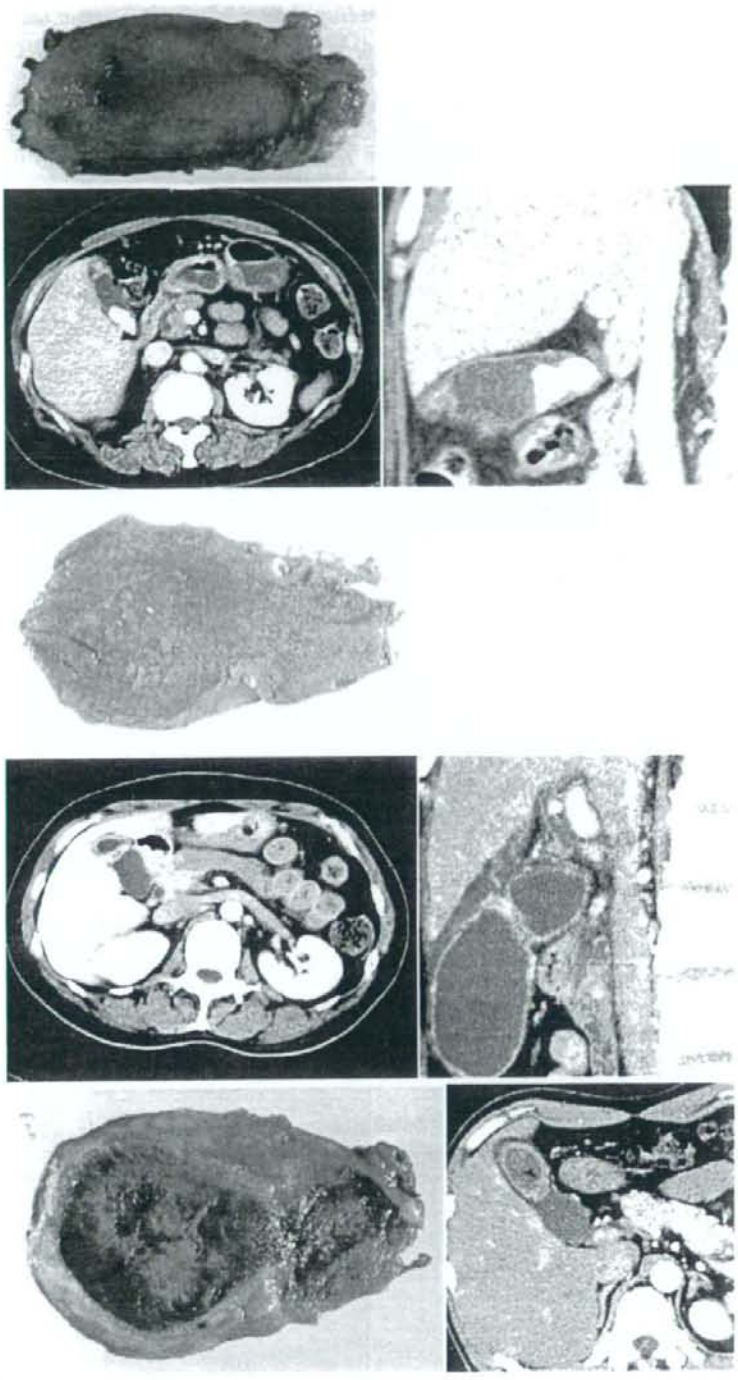


Fig. 3a–c. Findings in adenomyomatosis. **a** Fundal type: circumscribed hypertrophy of the fundus of the gallbladder with Rokitansky-Aschoff sinus. Case complicated by cholecystolithiasis. *Upper,* resected specimen; *lower left,* abdominal computed tomography (CT; cross section); *lower right,* abdominal CT (sagittal section). **b** Segmental type: hypertrophy circumscribing the gallbladder with Rokitansky-Aschoff sinus. *Upper,* resected specimen; *lower left,* abdominal computed tomography (CT; cross section); *lower right,* abdominal CT (sagittal section). **c** Diffuse (segmental-diffuse) type: hypertrophy and Rokitansky-Aschoff sinus from the gallbladder body to the fundus. Gallstones in the fundus. *Left,* resected specimen; *right,* abdominal CT