

cancer tissues and corresponding noncancerous tissues. Promoter hypermethylation was detected in 20 (62.5%) of the 32 primary pancreatic cancer tissues and in only two of the corresponding noncancerous tissues (Figure 1). To confirm the methylation of the RUNX3 promoter region, genomic bisulphite-treated DNA of primary pancreatic cancer tissues, which showed methylation by MSP, were sequenced. Every case showed at least one methylated CpG island of the sequenced fragments. A representative case is shown in Figure 2.

Mutational analysis of RUNX3 in pancreatic cancer tissues

To investigate the mutation status of this gene, RT-PCR-SSCP analysis was performed. We could not see any aberrant bands

(Figure 3). No mutations or polymorphisms were detected in the 32 pancreatic cancer tissues. As we used the bulk frozen samples, normal cells such as fibrosis cells were contaminated in the tumour tissues, making it difficult to identify aberrant bands.

Statistical analysis of clinicopathological data and our findings

Subsequently, we analysed the correlation between the clinicopathological data and results of our findings. Table 2 shows the correlation between the clinicopathological data and methylation status. Interestingly, RUNX3 hypermethylation was significantly correlated with a worse prognosis ($P=0.0143$) (Figure 4). No other correlation with any clinicopathological parameter was found.

To evaluate the value of RUNX3 methylation as an independent prognostic determinant, multivariate analysis was performed with prognostic factors that had been found to be significant by univariate analyses. The analysis identified lymph node metastasis, invasion of retroperitoneal tissue, and hypermethylation of RUNX3 gene as the variables for independently predicting overall survival (Table 3).

DISCUSSION

Transforming growth factor- β plays a key role in regulating the growth and differentiation of many cell types. In TGF- β 1-null

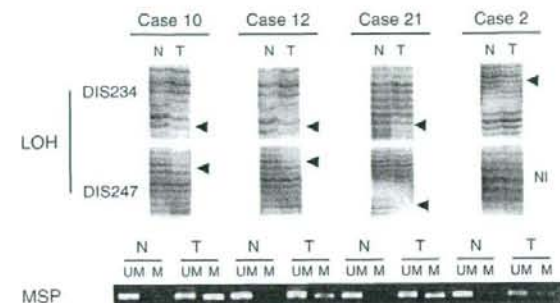


Figure 1 Representative results of LOH and MSP in cases 10, 12, 21, and 2. In the analysis of LOH at RUNX3 locus, cases 10, 12, and 21 showed allelic imbalance at DIS234 as well as at DIS247 (arrowheads). Case 2 showed allelic imbalance at DIS234 (arrowhead), but the DIS247 was not informative (NI). Promoter hypermethylation was observed in the DNA extracted from tumour tissue (T). In noncancerous samples (N), a methylation band was not seen in any lane. All four cases showed both LOH and promoter hypermethylation. These results indicated that biallelic inactivation (LOH at 1p36 + methylation) caused the inactivation of RUNX3 in pancreatic cancer. LOH, loss of heterozygosity; MSP = methylation-specific PCR; RUNX3 = human runt-related transcription factor 3 gene.

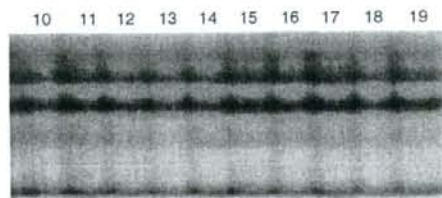


Figure 3 RT-PCR-SSCP analysis of RUNX3 in pancreatic cancer tissues. Representative results (cases 10–19) of RT-PCR-SSCP analysis using F2–R2 primer set. There were no aberrant bands in all cases. RT-PCR-SSCP = RT-PCR single-strand conformation polymorphism; RUNX3 = human runt-related transcription factor 3 gene.

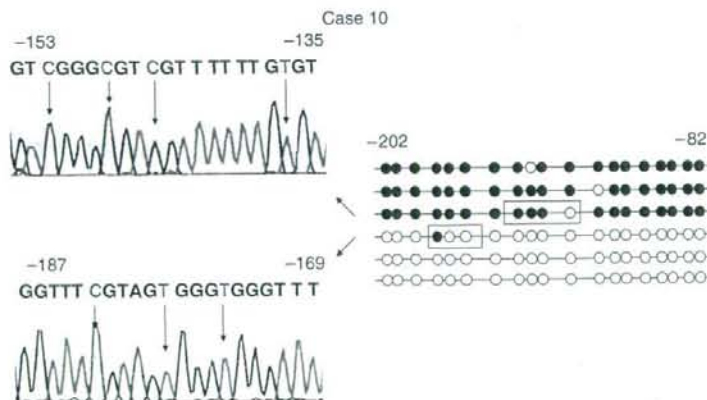


Figure 2 Sequence analysis of bisulphite-treated DNA from tumour sample of case 10 in RUNX3 promoter region. Methylation status of the 19 CpG islands between -82 and -202 from the transcription-initiation site of RUNX3 exon 1 is shown. The fragment was PCR amplified and subcloned into TA cloning vector. Closed circle indicates methylated CpG island, open circle indicates unmethylated CpG island. Each group of six clones showed a different methylation status. Arrows below the sequence indicate CpG islands. The Cs indicate methylated CpG islands. The Ts were converted from C by bisulphite treatment, indicating unmethylated CpG islands. RUNX3 = human runt-related transcription factor 3 gene.

Table 1 Clinicopathological features and results of RUNX3 alterations in pancreatic cancer tissues

Case	Gender	Location	Stage ^b	Pathology	Hypermethylation		LOH ^a	
					N	T	DIS234	DIS247
1	M	Ph	III	Tub. poor	—	—	●	●
2	F	Ph	IVa	Tub. mod	—	—	○	NI
3	M	Ph	IVb	Tub. mod	—	M	●	●
4	F	Ph	III	Tub. mod	—	—	●	NI
5	F	Phb	III	Anap. duc	—	—	NI	●
6	F	Ph	IVa	Tub. well	M	M	●	●
7	M	Ph	III	Tub.	—	M	NI	●
8	F	Ph	IVb	Tub.	—	M	●	●
9	M	Ph	IVb	Tub. mod	—	M	○	●
10	F	Ph	IVa	Tub. mod	—	M	○	○
11	M	Ph	IVb	Tub. mod	M	M	○	NI
12	M	Pb	III	Tub. poor	—	M	○	○
13	F	Ph	IVa	Tub. mod	—	—	●	●
14	M	Ph	IVa	Tub. mod	—	M	NI	●
15	M	Ph	IVb	Tub. mod	—	—	NI	●
16	M	Ph	IVb	Tub. poor	—	M	●	●
17	M	Ph	IVb	Tub. mod	—	—	●	○
18	M	Ph	IVb	Tub. mod	—	M	○	NI
19	M	Ph	IVb	Undifferentiated	—	M	●	○
20	F	Ph	IVb	Tub. mod	—	—	●	●
21	F	Phbt	IVb	Tub. mod	—	M	●	○
22	M	Ph	III	Acinar cell ca.	—	M	○	○
23	F	Ph	IVb	Tub.	—	—	●	●
24	F	Phb	IVa	Tub. mod	—	—	NI	NI
25	F	Ph	IVa	Tub. poor	—	M	NI	●
26	M	Pb	IVa	Tub. well	—	M	NI	NI
27	F	Ph	III	Tub. mod	—	M	●	NI
28	M	Ph	IVa	Tub. mod	—	M	●	NI
29	M	Ph	III	Tub. mod	—	—	●	NI
30	M	Ph	IVa	Tub. well	—	—	●	NI
31	M	Pb	IVa	Tub. poor	—	M	NI	●
32	F	Ph	IVa	Tub. mod	—	M	○	○
					2/32 (6.3%)	20/32 (62.5%)	8/32 (25%)	7/32 (21.9%)
							LOH ^a : 11/32 (34.3%)	

^aAnap. duc = anaplastic ductal adenocarcinoma; F = female; LOH = loss of heterozygosity; M = male; M = methylated; mod = moderately differentiated adenocarcinoma; N = normal tissue; NI = not informative; Pb = pancreatic body; Pt = pancreatic tail; poor = poorly differentiated adenocarcinoma; Ph = pancreatic head; T = tumour tissue; tub = tubular adenocarcinoma; well = well-differentiated adenocarcinoma; —, unmethylated; open circle = LOH detected; closed circle = retention of heterozygosity; LOH^a = cases in which LOH was detected in at least one locus. ^bThe stage classification was performed according to the Pancreatic Cancer Study Group of Japan.

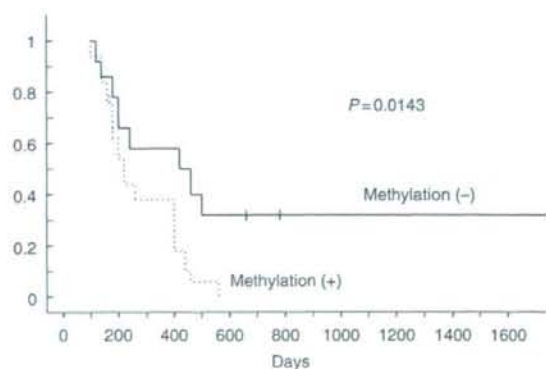


Figure 4 Survival stratified by methylation status in primary pancreatic cancer. RUNX3 hypermethylation was significantly correlated with a worse prognosis ($P=0.0143$). RUNX3 = human runt-related transcription factor 3 gene.

animals, proliferation of the gastric epithelium is stimulated and hyperplasia occurs (Crawford *et al*, 1998). TGF- β is known to be a potent inhibitor of pancreatic acinar and duct cell proliferation *in vitro* (Bisgaard and Thorgeirsson, 1991; Logsdon *et al*, 1992).

RUNX3 is a runt domain transcription factor involved in this signalling pathway. RUNX3 protein binds with the Smad2 and Smad3 proteins. Recently, it has been reported that RUNX3 was one of the tumour suppressor genes in gastric cancer and testicular yolk sac tumour. Runx3-null mice reportedly develop hyperplasia of the gastric mucosa through activation of cellular proliferation and suppression of apoptosis in epithelial cells (Li *et al*, 2002). Interestingly, 1p36, where RUNX3 exists, is a region commonly deleted in a wide variety of human carcinomas, including pancreatic cancer. To date, there are many reports regarding the TGF- β signalling pathway in pancreatic cancer (e.g. TGF- β receptor II, Smad2 and Smad4), but only a few deal with this gene's alterations in pancreatic cancer (Li *et al*, 2004; Wada *et al*, 2004). Moreover, there are no reports regarding primary pancreatic cancer. Our study further supports a role for RUNX3 in pancreatic cancer.

The 1p36 region is believed to harbour tumour suppressor genes, because previous studies identified frequent allelic imbalance at 1p36 in various types of human cancers (Schwab *et al*, 1996). RIZ1 and p73 genes are located on 1p36, and LOH was detected at each gene locus in pancreatic cancer (Sakurada *et al*, 2001; Sphyris *et al*, 2004). It is thought that these are one of the tumour suppressor genes in pancreatic cancer, and we think that RUNX3 may also be a candidate.

Previously, Wada *et al* (2004) reported that nine of 12 pancreatic cancer cell lines exhibited no expression of RUNX3 by both

Table 2 Clinicopathological features and results of RUNX3 hypermethylation in pancreatic cancer tissues

Variable	No. of cases	Hypermethylation		P ^a
		+	-	
Age				
<60	10	5	5	0.325
≥60	22	15	7	
Gender				
M	18	13	5	0.198
F	14	7	7	
Tumour size				
TS1	5	2	3	>0.9999
≥TS2	27	18	9	
S				
-	18	11	7	0.854
+	14	9	5	
RP				
-	10	6	4	0.844
+	22	14	8	
CH				
-	13	9	4	0.515
+	19	11	8	
DU				
-	21	13	8	0.923
+	11	7	4	
PV				
-	12	8	4	0.706
+	20	12	8	
A				
-	27	16	11	0.379
+	5	4	1	
PL				
-	27	16	11	0.379
+	5	4	1	
DPM				
-	26	15	11	0.242
+	6	5	1	
N				
0	14	8	6	0.581
1	18	12	6	
Differentiation				
Mod	21	12	9	0.241
Poor	6	5	1	

^aAnalysed by Fisher's exact test or χ^2 test for independence. ^bTumour size according to the Classification of Pancreatic Carcinoma; A = arterial invasion; CH = choledochal invasion; DPM = dissected peripancreatic tissue margin; DU = duodenal invasion; F = female; PL = peripancreatic nerve plexus invasion; M = male; mod = moderately differentiated adenocarcinoma; N = lymph node metastasis; poor = poorly differentiated adenocarcinoma; pTNM = pathological TNM; PV = portal vein invasion; RP = retroperitoneal invasion; S = serosal invasion. ^cClassified according to the classification of The General Rules for the Clinical and Pathological Study of Primary Pancreatic Cancer, April 2002, Pancreatic Cancer Study Group of Japan.

Table 3 Multivariate analysis of patients with pancreatic cancer

Variable	Odds ratio	95% CI	P
Tumour size (≥2.0 cm)	1.995	0.639–6.226	0.2342
Lymph node metastasis	2.388	1.026–5.561	0.0435*
Invasion of retroperitoneal tissue (d.p.m.)	5.486	1.409–21.358	0.0141*
Invasion of plexus nerve (PI)	1.759	0.591–5.239	0.3103
Hypermethylation	3.157	1.226–8.130	0.0172*

*Statistical significance. CI = confidence interval.

northern blot analysis and RT-PCR. All of the nine cell lines showed methylation of the promoter CpG island of the gene. Moreover, hemizygous deletion of RUNX3, as detected by fluorescence *in situ* hybridisation, was found in most of the cell lines that lacked RUNX3 expression. Our results using primary pancreatic cancer tissue were compatible with their findings.

Li et al (2004) reported that RUNX3 expression was low-to-absent in normal pancreatic tissues, but increased in a third of cancer tissues by RT-PCR and immunohistochemistry. RUNX3 expression was present only in islets of the normal pancreas. They

also found that all metastases of pancreatic cancer tissues were devoid of or displayed only very faint RUNX3 expression by immunostaining.

Some groups have advocated islet cells as the cells of origin of pancreatic ductal adenocarcinoma (Pour et al, 2003). This would mean that the islet cells in pancreatic tissue are the tissue-specific stem cells in which cancer cells begin from the alteration in the oncogenes or tumour suppressor genes. RUNX3 is expressed in the tissue-specific stem cells, and only in islet cells in normal tissue. When cancer tissue has grown from the tissue-specific stem cells, the cancer cells express the RUNX3 protein. Some cancer tissues do not express RUNX3. In those cancer cells, RUNX3 gene is methylated. In cases with metastatic lesions, more aggressive tumour cells from the original lesion exist, such as RUNX3-methylated cells. Hence, the metastatic pancreatic cancer cells do not express RUNX3 gene.

Thus, it may be hypothesised that there is indeed loss of RUNX3 expression by promoter hypermethylation or LOH in some

primary tumours compared with normal islets, and almost a complete loss in metastatic tumours. Our finding that the survival in methylated cases in RUNX3 gene was significantly worse than that in unmethylated patients is compatible with this hypothesis, although pointing to a tumour suppressor role for RUNX3 in pancreatic cancer.

Nine of 11 LOH detected cases had hypermethylation of the RUNX3 promoter region. These findings imply that silencing of RUNX3 occurred biallelically. Complete silencing of this gene leads to the progression of cancer, and then relates to the worse prognosis.

In conclusion, we have clearly demonstrated for the first time that RUNX3 is frequently methylated in primary pancreatic cancer tissues, frequent hemizygous deletion occurs at its locus in 1p36, and RUNX3-inactivated cases showed worse survival. We propose that inactivation of RUNX3 plays an important role in alteration of the TGF- β signalling pathway and in the tumorigenesis of pancreatic cancer.

REFERENCES

- Biggaard HC, Thorgeirsson SS (1991) Evidence for a common cell of origin for primitive epithelial cells isolated from rat liver and pancreas. *J Cell Physiol* 147: 333–343
- Crawford SE, Stellmach V, Murphy-Ullrich JE, Ribeiro SM, Lawler J, Hynes RO, Boivin GP, Bouck N (1998) Thrombospondin-1 is a major activator of TGF- β 1 *in vivo*. *Cell* 93: 1159–1170
- Ito Y, Miyazono K (2003) RUNX transcription factors as key targets of TGF- β signaling. *Curr Opin Genet Dev* 13: 43–47
- Japan Pancreatic Society (2003) *Classification of Pancreatic Carcinoma* 2nd English edn, Tokyo: Kanehara
- Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, Thun MJ (2007) Cancer statistics, 2007. *CA Cancer J Clin* 57: 43–66
- Kern SE, Hruban RH, Hidalgo M, Yeo CJ (2002) An introduction to pancreatic adenocarcinoma genetics, pathology and therapy. *Cancer Biol Ther* 1: 607–613
- Li J, Kleff J, Guweidhi A, Esposito I, Berberat PO, Giese T, Büchler MW, Friess H (2004) RUNX3 expression in primary and metastatic pancreatic cancer. *J Clin Pathol* 57: 294–299
- Li QL, Ito K, Sakakura C, Fukamachi H, Inoue K, Chi XZ, Lee KY, Nomura S, Lee CW, Han SB, Kim HM, Kim WJ, Yamamoto H, Yamashita N, Yano T, Ikeda T, Itohara S, Inazawa J, Abe T, Hagiwara A, Yamagishi H, Ooe A, Kaneda A, Sugimura T, Ushijima T, Bae SC, Ito Y (2002) Causal relationship between the loss of RUNX3 expression and gastric cancer. *Cell* 109: 113–124
- Logsdon CD, Keyes L, Beauchamp RD (1992) Transforming growth factor- β (TGF- β 1) inhibits pancreatic acinar cell growth. *Am J Physiol* 262: 364–368
- Loukopoulos P, Shibata T, Katoh H, Kokubu A, Sakamoto M, Yamazaki K, Kosuge T, Kanai Y, Hosoda F, Imoto I, Ohki M, Inazawa J, Hirohashi S (2007) Genome-wide array-based comparative genomic hybridization analysis of pancreatic adenocarcinoma: identification of genetic indicators that predict patient outcome. *Cancer Sci* 98: 392–400
- Massagu J, Blain SW, Lo RS (2000) TGF β signaling in growth control, cancer, and heritable disorders. *Cell* 103: 295–309
- Miyazono K, Suzuki H, Imamura T (2003) Regulation of TGF- β signaling and its roles in progression of tumors. *Cancer Sci* 94: 230–234
- Nowak NJ, Gaile D, Conroy JM, McQuaid D, Cowell J, Carter R, Goggins MG, Hruban RH, Maitra A (2005) Genome-wide aberrations in pancreatic adenocarcinoma. *Cancer Genet Cytogenet* 161: 36–50
- Ozawa F, Friess H, Tempia-Caliera A, Kleff J, Büchler MW (2001) Growth factors and their receptors in pancreatic cancer. *Teratog Carcinog Mutagen* 21: 27–44
- Pour PM, Pandey KK, Batra SK (2003) What is the origin of pancreatic adenocarcinoma? *Mol Cancer* 2: 13
- Sakurada K, Furukawa T, Kato Y, Kayama T, Huang S, Horii A (2001) RIZ, the retinoblastoma protein interacting zinc finger gene, is mutated in genetically unstable cancers of the pancreas, stomach, and colorectum. *Genes Chromosomes Cancer* 30: 207–211
- Schwab M, Pram C, Amler LC (1996) Genomic instability in 1p and human malignancies. *Genes Chromosomes Cancer* 16: 211–229
- Sphyris N, Morris RG, Harrison DJ (2004) Induction of p21 and nuclear accumulation of TAp73alpha and c-abl during apoptosis of cisplatin-treated primary pancreatic acinar cells. *Int J Oncol* 25: 1661–1670
- Tokumaru Y, Nomoto S, Jerónimo C, Henrique R, Harden S, Trink B, Sidransky D (2003) Biallelic inactivation of the RIZ1 gene in human gastric cancer. *Oncogene* 22: 6954–6958
- Wada M, Yazumi S, Takaishi S, Hasegawa K, Sawada M, Tanaka H, Ida H, Sakakura C, Ito K, Ito Y, Chiba T (2004) Frequent loss of RUNX3 gene expression in human bile duct and pancreatic cancer cell lines. *Oncogene* 23: 2401–2407

A gastrin transcript expressed in gastrointestinal cancer cells contains an internal ribosome entry site

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As the hormone gastrin promotes gastrointestinal (GI) cancer progression by triggering survival pathways, regulation of gastrin expression at the translational level was explored. Sequence within the 5' untranslated region of a gastrin transcript expressed in GI cancer cells was investigated, then cloned into a bicistronic vector upstream of firefly luciferase and transfected into a series of GI cancer cell lines. Firefly luciferase activity was measured relative to that of a cap-dependent *Renilla* luciferase. A gastrin transcript that was different from that described in Ensembl was expressed in GI cancer cells. Its transcription appears to be initiated within the region designated as the gene's first intron. In GI cancer cells transfected with the bicistronic construct, firefly luciferase activity increased 8–15-fold compared with the control vector, and there was a further induction of the signal (up to 25-fold) following exposure of the cells to genotoxic stress or hypoxia, suggesting that the sequence acts as an internal ribosome entry site. These data suggest that the gastrin transcript within GI cancer cells contains an internal ribosome entry site that may allow continued expression of gastrin peptides when normal translational mechanisms are inactive, such as in hypoxia, thereby promoting cancer cell survival. *British Journal of Cancer* (2008) **98**, 1696–1703. doi:10.1038/sj.bjc.6604326 www.bjcancer.com

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Keywords: gastrin; translation; gastrointestinal; internal ribosome entry

Gastrin is normally expressed in G cells of the stomach antrum and regulates both acid secretion and proliferation of gastric mucosal cells (Watson *et al*, 2006). The gastrin gene (*GAST*) is a 4-kb unit consisting of three exons and two introns with the gastrin polypeptide encoded by sequence within exons 2 and 3 (<http://www.ensembl.org/index.html>). Two different gastrin transcripts have been described in the literature. The transcript given in the Ensembl database was described in human gastrinomas and is a 434-bp transcript incorporating sequence from exon 1 (Ito *et al*, 1984; Wiborg *et al*, 1984). However, another transcript was identified in the gastric antrum that has a transcription start site 111 bp upstream of the start codon (Kato *et al*, 1983). Thus, the 5' untranslated regions (5'UTRs) of the Ensembl and alternative transcripts are different.

Gastrin upregulation has been shown at both the gene and protein levels in a number of gastrointestinal (GI) (Goetze *et al*, 2000; Mukawa *et al*, 2005; Hur *et al*, 2006) and non-GI cancers (Rehfeld *et al*, 1989; van Solinge *et al*, 1993). At the transcriptional level in GI cancer, gastrin upregulation may be a result of mutational events, for example in the *APC* (adenomatous polyposis coli) or *k-ras* genes (Nakata *et al*, 1998; Koh *et al*, 2000); engagement of the EGF (epidermal growth factor) receptor

(Merchant *et al*, 1991); inflammatory events mediated directly by cytokines, such as those associated with *Helicobacter pylori* infection (Suzuki *et al*, 2001; Beales, 2004); or direct activation by certain pathogenicity factors expressed by *Helicobacter pylori* (Rieder *et al*, 2005).

Expression of a number of genes that promote cancer cell survival has been shown to be regulated at the translational level (Pickering and Willis, 2005; Sontheimer and Carthew, 2005). One mechanism, involving the presence of an internal ribosome entry site (IRES) within the 5'UTR of the transcript, may have evolved to allow continued expression of key proteins involved in cell survival during cellular stress (Bushell *et al*, 2004; Holcik and Sonenberg, 2005) when conventional cap-mediated translation is reduced. However, it may also contribute to cancer cell survival as IRESs have been identified in the transcripts of genes that increase proliferation, protect against apoptosis and promote angiogenesis (Vagner *et al*, 1995; Miller *et al*, 1998; Stoneley *et al*, 2000a; Coldwell *et al*, 2001).

Gastrin plays an important role in establishing and supporting the growth of a range of GI tumours (Ferrand and Wang, 2006; Watson *et al*, 2006). As well as acting as a growth hormone, it has well-documented pro-angiogenic (Clarke *et al*, 2006) and anti-apoptotic properties (Konturek *et al*, 2003; Harris *et al*, 2004; Ramamoorthy *et al*, 2004). We have previously used RNAi to downregulate gastrin expression at the gene level and observed a rapid loss of the transcript, but also a delayed downregulation of the endogenous protein compared with GFP-tagged gastrin encoded by a transcript lacking the gastrin 5'UTR (Grabowska *et al*, 2007). This raised the possibility that gastrin expression may be regulated translationally in a manner dependent on the 5'UTR

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Effects of tumor selective replication-competent herpes viruses in combination with gemcitabine on pancreatic cancer

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Abstract

Purpose Pancreatic cancer still has a poor prognosis, even if aggressive therapy is pursued. Currently, new modalities of oncolytic virus therapy are being tested against this cancer. The combination of one of two representative mutant herpes simplex viruses (R3616: γ_1 34.5 inactivated, hrR3: UL39 inactivated) with a standard anti-pancreatic cancer chemotherapy drug (gemcitabine), was investigated in this study.

Experimental design The intracellular concentration of ribonucleotide reductase was estimated by Western blotting. The effect of gemcitabine on viral replication and the total cytotoxic effect of the combination therapy were investigated on pancreatic cancer cell lines. We compared the results of two oncolytic viruses, R3616 and hrR3. A mouse model of pancreatic cancer with peritoneal dissemination was used to evaluate the in vivo effect of the combination therapy.

Results Although the replication of both viruses was inhibited by gemcitabine, the combination caused more tumor cell cytotoxicity than did virus alone in vitro. The results with R3616 were more striking. Although the difference was not statistically significant, R3616 with gemcitabine had a greater effect than did R3616 alone, while hrR3 with gemcitabine had a weaker effect than did hrR3 alone in vivo experiments.

Conclusion The combination of oncolytic virus with gemcitabine is a promising new strategy against advanced

pancreatic cancer. Each virus has different functional characteristics, and can affect the results of the combination of viruses and chemotherapy drugs. The results indicate that there is a complicated interaction among viruses, cells, and chemotherapy drugs and that the best combination of oncolytic virus and chemotherapeutic agents should be studied more extensively before embarking on a clinical trial.

Keywords Herpes oncolytic virus · Pancreatic cancer · Gemcitabine · Combination therapy

Introduction

Pancreatic cancer is a disease with an extremely poor prognosis. Surgical therapy for pancreatic cancer is still insufficient to cure most patients [1]. Recently gemcitabine has shown a modest survival advantage over 5-fluorouracil (5-FU) in patients with this cancer [2]. Gemcitabine has become one of the standard chemotherapy drugs against pancreatic cancer but more effective therapies must be devised in order to significantly improve survival. Oncolytic virus therapy has been highly trusted as a new type of therapy for advanced incurable pancreatic cancer, and may provide some clinical benefit to those patients in the near future. Currently, clinical trials using oncolytic viruses have been started against many types of cancer in world-wide [3], such as brain cancer [4, 5], prostate cancer [6, 7], pancreatic cancer [8], breast cancer [9], and head and neck cancer [10, 11]. This study investigated the possibility of combination therapy using gemcitabine and two herpes mutant oncolytic viruses (R3616 and hrR3) against pancreatic cancer.

Gemcitabine (difluorodeoxycytidine; dFdC) is intracellularly phosphorylated to difluorodeoxycytidine diphosphate (dFdCDP) and difluorodeoxycytidine triphosphate

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(dFdCTP). dFdCTP competes with deoxycytidine triphosphate (dCTP) for incorporation into DNA, and DNA synthesis is inhibited [2, 12, 13]. In addition, dFdCDP acts as an inhibitor of ribonucleotide reductase (RR) in cells, which in turn causes a major decrease in the dCTP pool. Therefore, gemcitabine reduces the activity of RR in cancer cell lines [14] (Fig. 1a). However, some cells have been known to acquire chemoresistance to gemcitabine due to over expression of RR [15–21].

R3616 and hrR3 are genetically engineered herpes simplex viruses [3]. R3616 lacks the $\gamma_{134.5}$ gene that produces the ICP34.5 protein. Replication of R3616 is severely restricted in normal cells, because the expression of ICP34.5 in normal cells prevent a protein shutoff mechanism that is associated with eIF2 α dephosphorylation through the protein kinase receptor (PKR). Most cancer cells lose this normal protein shutoff mechanism so that viral replication can proceed, which induces the virally infected cells to undergo apoptosis to protect the integrity of the cell's DNA and block viral replication [3, 22–24]. hrR3 lacks the UL39 gene that produces the ICP6 proteins (viral RR), a key enzyme in the biosynthesis of DNA in all prokaryotic and eukaryotic cells. The viral replication of hrR3 is severely restricted in cells that have high levels of holding proteins involved in nucleic acid synthesis such as cancer cells [25, 26] (Fig. 1b).

We investigated the effect of tumor-selective, replication-competent herpes viruses (R3616 and hrR3) against pancreatic cancer under the same conditions in which gemcitabine effects cancer cells. Our major concern was how gemcitabine may interrupt viral replication, and whether the combination of an oncolytic virus with gemcitabine can significantly improve anti-pancreatic cancer therapy.

Materials and methods

Viruses and cells

R3616 was kindly provided by Bernard Roizman Sc. D (University of Chicago, Chicago, IL, USA) and hrR3 was kindly provided by Sandra K. Weller Ph.D. (University of Connecticut, Storrs, CT, USA). SW1990, derived from a human pancreatic carcinoma, was kindly provided by Dr. T. Sawada (First Department of Surgery, Osaka City University, Osaka, Japan). CAPAN 1, also derived from a human pancreatic carcinoma, was obtained from the Japanese Cancer Research Resources Bank, Tokyo, Japan. PACA2, another cell line derived from a human pancreatic carcinoma, was obtained from the American Type Culture Collection, Manassas, VA, USA. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and 1% penicillin/streptomycin at 37°C (Sigma, Tokyo, Japan).

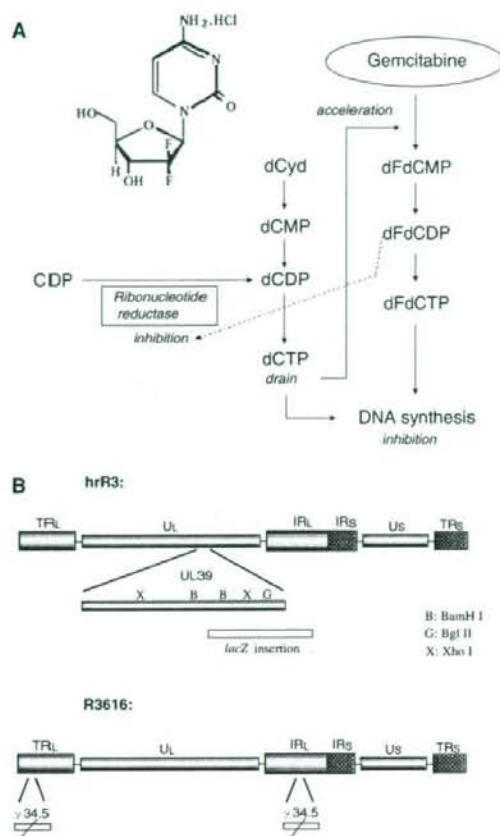


Fig. 1 a Gemcitabine structure and pathway. Gemcitabine HCl is a nucleoside analog that exhibits anti-tumor activity. Gemcitabine HCl is 2'-deoxy-2', 2'-difluorocytidine monohydrochloride (β -isomer). The empirical formula for gemcitabine HCl is C₉H₁₁F₂N₃O₄ × HCl. It has a molecular weight of 299.66. Gemcitabine is metabolized intracellularly by nucleoside kinases to the active diphosphate (dFdCDP) and triphosphate (dFdCTP) nucleosides. b Schematic illustration of hrR3 and R3616. hrR3 is a mutated herpes simplex virus (HSV) that has the LacZ gene inserted into the site of UL39 (ICP6), causing inactivation of ribonucleotide reductase activity that is associated with UL39. Ribonucleotide reductase is a key enzyme for viral DNA synthesis. R3616 is a mutated HSV that has a deletion of both $\gamma_{134.5}$ genes. The $\gamma_{134.5}$ gene produces ICP 34.5 that dephosphorylates eIF2 α -phosphate to permit continued viral protein synthesis. Those mutated HSVs replicate and destroy only the cancer cells

Western blot assay

A total of 10⁶ cells were harvested and rinsed twice with phosphate-buffered saline, pH 7.4. Cell extracts were prepared with lysis buffer (20 mM Tris, pH 7.5, 0.1% Triton-X, 0.5% deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, and 10 μ g/ml leupeptin) and clarified by

centrifugation at 12,000g, for 15 min, at 4°C. Cell lysates containing equal amounts of protein as determined by a BCA assay kit were electrophoresed on a NuPAGE, Novex 4–12% Bis-Tris Gel (Invitrogen, Carlsbad, CA, USA), and the resolved proteins were transferred to PVDF membranes (Invitrogen). The membranes were blocked with 5% nonfat milk overnight at room temperature, and incubated with 0.2 µg/ml human anti-RRM1 antibody (CHEMICON International, Temecula, CA, USA) for 1 h. RRM1 was detected using an enhanced chemiluminescence (ECL) system following the manufacturer's instructions (Amersham Life Science, Uppsala, Sweden). β -actin also was detected on the same membrane to serve as a control for the amount of protein loaded.

Cytotoxic assay

Gemcitabine and viral-induced cytotoxicity assays were performed using the MTT assay as previously described [27, 28]. Briefly, 10^6 cells were plated in a 10-cm plate and 10 µg/ml of gemcitabine was added. After 24 h, a replication-competent virus (R3616 or hrR3) was added at multiplicity of infection (MOI) values ranging from 0.01 to 10 and incubated for an additional 48 h. The number of surviving cells was quantified by a colorimetric MTT assay. The results, expressed as mean \pm SD of four samples, were compared with the results from the cytotoxicity assays of gemcitabine alone and the virus alone. Statistical significance was determined by the two-sided Student's *t*-test using SPSS (SPSS, Chicago, IL, USA).

Viral replication assay

Viral replication assays were performed as described [28, 29]. Briefly, 10^6 cells were plated in a 10-cm plate and 10 µg/ml of gemcitabine was added. After 24 h, replication competent viruses (R3616 or hrR3) were added at MOI of 2. Forty-eight hours after infection, the supernatant and cells were harvested, exposed to three freeze-thaw cycles to release the virions, and titered. The results were compared with the assays of viral replication without gemcitabine.

Animal studies

Mice (6-week-old females BALB/c nu/nu) were obtained from the Charles River Japan, Yokohama, Japan. Animal studies were performed in accordance with the guidelines issued by the Nagoya University Animal Center. The mice, used in a peritoneal-disseminated carcinoma model, were injected with 10^6 PACA2 cells into the intraperitoneal cavity. The condition of the animals was checked once or twice a day for the duration of the study. The mice were divided randomly into six groups (A–F). Group A ($n = 10$), group D ($n = 10$), and group E ($n = 10$) were injected with 1 mg of

gemcitabine into the intraperitoneal cavity on day 14 after the injection of the PACA2 cells. The mice in groups A and B ($n = 10$) each were injected with 10^6 particles of R3616 on day 15 after the injection of the PACA2 cells. Group C ($n = 10$) and group D ($n = 10$) were injected with 10^6 particles of hrR3 on day 15 after the injection of the PACA2 cells. Group F ($n = 10$) was the control group, which was injected with only PACA2 cells into the intraperitoneal cavity.

Statistical differences between groups were determined by the log-rank test with the use of JMP 5.0 software (SAS Inc., Cary, NC, USA). $P < 0.05$ was considered statistically significant.

Results

Expression of RRM1 by Western blotting

As previously reported by many researchers on their papers, overexpression of ribonucleotide reductase subunit 1 (RRM1) is associated with chemoresistance to gemcitabine [15–17]. We examined the intensity of RRM1 protein expression in Capan1, PACA2, and SW1990 cells (Fig. 2). The intensity of RRM1 expression in the PACA2 cells was greater than in the other cell lines. The results from many previous related papers regarding chemoresistance to gemcitabine, indicated that PACA2 cells might have the highest potential of chemo resistance to gemcitabine among the three cell lines.

Comparison of cytotoxic assays between hrR3 and R3616, with or without gemcitabine

We compared the cytotoxicity of R3616 (γ 34.5 deficiency) and hrR3 (ICP6: RR gene deficiency) viruses' combination with gemcitabine by the MTT assay (Fig. 3). With both R3616 and hrR3, the cytotoxicity was increased by their

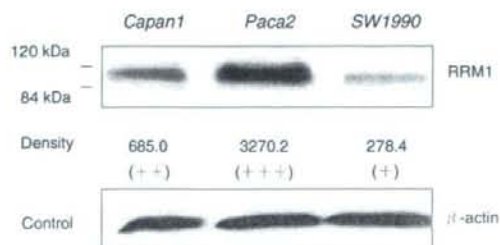
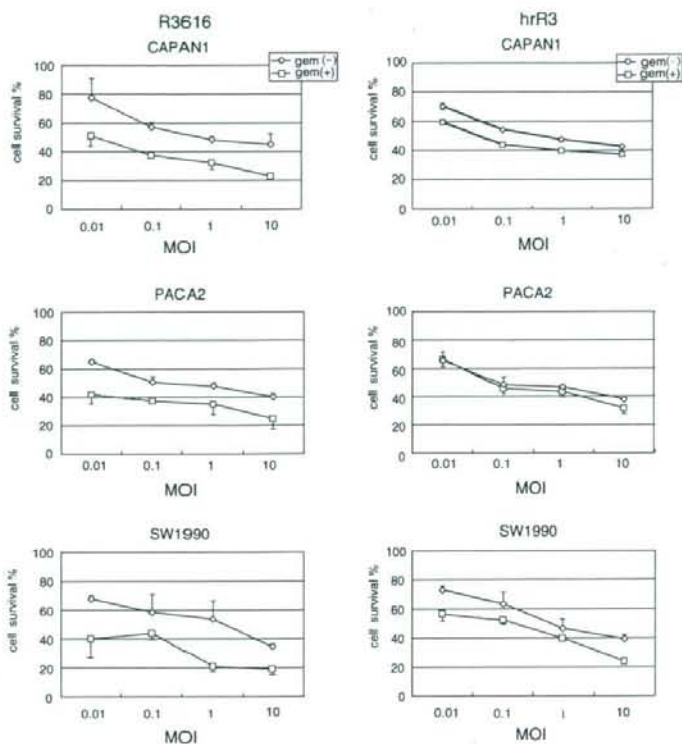


Fig. 2 Expression of ribonucleotide reductase M1 (RRM1) by Western blotting. PACA2 cells expressed the most ribonucleotide reductase M1 (RRM1) by Western blot assays among three pancreatic cancer cell lines tested. β -actin served as a control for the amount of protein loaded in each lane

Fig. 3 Comparison of cytotoxic assays between hrR3 and R3616, with or without gemcitabine. For both R3616 (γ 34.5 gene inactivated) and hrR3 (ICP6: ribonucleotide reductase gene inactivated), the cytotoxicity was increased when combined with gemcitabine. However, there was a trend toward greater cytotoxicity with R3616 than with hrR3 against all cell lines



combination with gemcitabine, but the more significant increase in cytotoxicity was observed with R3616 than hrR3. On the other hand, PACA2 cells, which expressed the most RRM1 by a Western blot assay, had the lowest increase in cytotoxicity with the combination of hrR3 and gemcitabine.

Comparison of cytotoxic assays between gemcitabine alone and gemcitabine with low titer virus

We also compared the cytotoxicity between gemcitabine alone and gemcitabine with low titer virus by the MTT assay (Fig. 4). Of all cell lines, the combination of gemcitabine and an MOI 0.01 of R3616 showed more cytotoxic tendency than did gemcitabine alone ($P = 0.04$ on PACA2 cell line), while the combination of gemcitabine with an MOI 0.01 of hrR3 tend to be less cytotoxic than gemcitabine alone. PACA2 cells.

Comparison of viral replication between hrR3 and R3616, with or without gemcitabine

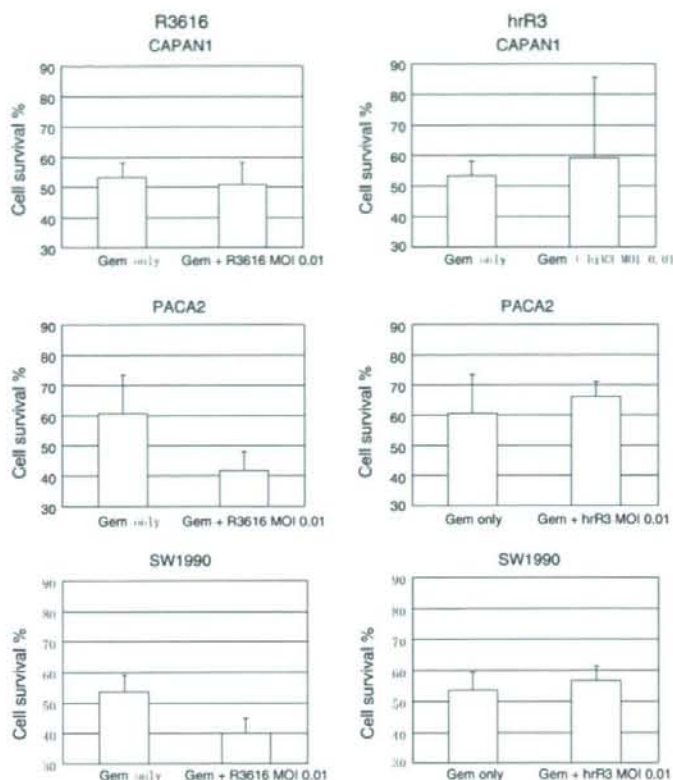
We compared the viral replication between R3616 and hrR3 in the presence of gemcitabine by the plaque-forming

assay (Fig. 5). The replication of both viruses was inhibited by gemcitabine. The titer of hrR3 declined more than did R3616 in combination with gemcitabine. The replication of hrR3 was inhibited by gemcitabine in all cell lines. PACA2 cells expressed the most RRM1 by Western blot assay, and hrR3 replicated more vigorously with gemcitabine in the PACA2 cells than in the other two cell lines, while R3616 was also inhibited by gemcitabine in all cell lines but with somewhat weaker inhibition comparing to hrR3.

Animal studies

Long-term survival (LTS: 100 days) was achieved in 60% of mice treated with an intraperitoneal injection of R3616 followed by gemcitabine (group A). Mice treated with an intraperitoneal injection of R3616 had only a 50% LTS (group B). Mice treated with hrR3 had a 30% LTS (group C). Mice treated with hrR3 followed by gemcitabine had a 20% LTS (group D). Mice treated with gemcitabine alone had only a 10% LTS (group E). All mice in the control group died within 60 days (group F) (Fig. 6). Statistical differences in the survival rates were determined by log-rank analyses (group A versus group F, $P = 0.0011$; group A versus group D, $P = 0.0078$; group E versus group F,

Fig. 4 Comparison of cytotoxic assays between gemcitabine alone and gemcitabine with a very low titer of virus. For all cell lines, the combination of gemcitabine and a very low titer (MOI 0.01) of R3616 tended to exhibit greater cytotoxicity than did gemcitabine alone ($P = 0.04$ on PACA2 cell line), on the other hand, the combination of gemcitabine and a very low titer (MOI 0.01) of hrR3 tended to be less cytotoxic than gemcitabine alone



$P = 0.006$; group B versus group D, $P = 0.0174$; group B versus group F, $P = 0.0049$). There were no other statistically significant differences between the other groups except for shown above. Although it was not significantly different, R3616 with gemcitabine tended to have a stronger effect than did R3616 alone, while hrR3 with gemcitabine tended to be weaker than hrR3 alone.

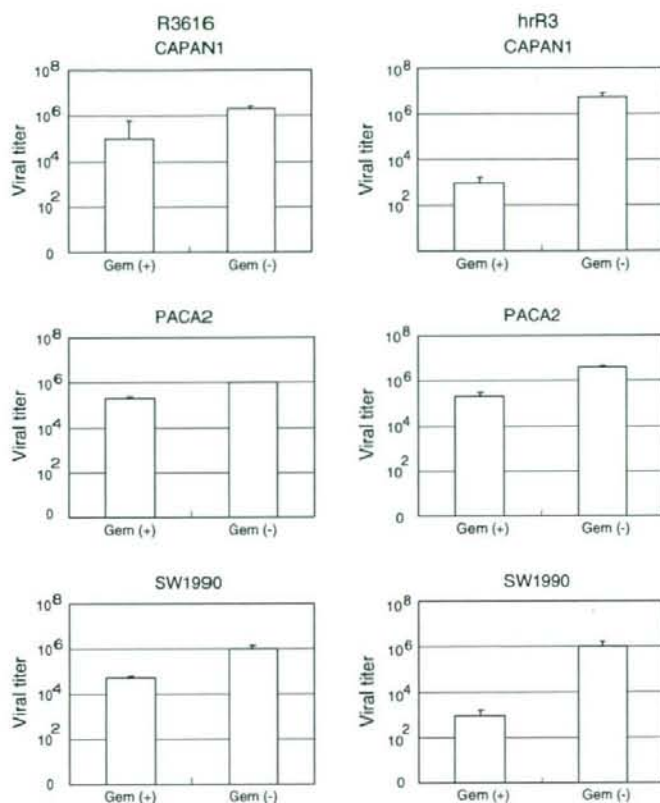
Discussion

In this study, we compared the efficacy of hrR3 or R3616 plus gemcitabine against pancreatic cancer. An *in vitro* cytotoxic assay indicated that R3616 plus gemcitabine caused a significant increase in the cell-killing effect in all three pancreatic cancer cell lines than did hrR3. We postulate that this result was due to the functional differences caused by each deleted viral gene. The viral replication of hrR3 might be more interrupted by the effect of gemcitabine than that of R3616, and this reduction might have been responsible for the slight decrease in the cell-killing effect

of hrR3. Cellular RR is important for viral replication especially for hrR3 that has no RR [3, 25–27, 29, 30]. Gemcitabine is well known to reduce the activity of cellular RR in cancer cell lines [14]. Therefore, it is a possible that the effect of gemcitabine was greater in combination with hrR3 than with R3616 reducing the replication and cytotoxicity of the viruses.

Interestingly, infection with hrR3 at a very low concentration (MOI 0.01) in the presence of gemcitabine caused less cytotoxic than did gemcitabine alone. This may be the result of the virus protecting the cancer cells from the apoptosis caused by gemcitabine. The virus itself has some anti-apoptotic effects on cells in order to protect the host cells from bursting too early and until the virus particles have matured. Although gemcitabine reduced the replication of hrR3, some viral anti-apoptosis genes might still have worked in the infected cells without the burst-cell effect that is caused by an abundance of mature viruses. The apoptosis mechanism might malfunction as a result of this low virus concentration, causing an anti-apoptotic effect against gemcitabine. This effect might apply not only to HSV, a critical

Fig. 5 Comparison of viral replication between hrR3 and R3616, with or without gemcitabine. The viral replication of both R3616 and hrR3 were inhibited by the presence of gemcitabine. This phenomenon was more prominent with hrR3 and gemcitabine than with R3616



consideration when using a viral vector or an oncolytic virus with chemotherapy drugs, because most viruses have such an anti-apoptosis gene. Examples include US3 and US5 in HSV [31, 32], and E1b 19 kDa in adenovirus [33]. Furthermore, several distinct viruses have been shown to develop mechanisms to block premature apoptosis of infected cells [34–36]. This phenomenon should be considered when using any viral vector for gene therapy or oncolytic virus therapy with chemotherapy drugs. In our opinion, the anti-apoptosis genes in a virus should be studied more intensively if future development of oncolytic virus therapy is to proceed.

PACA2 cells had the highest density of RR by Western assays and also the lowest cytotoxic effect from single agent gemcitabine among the three pancreatic cancer cell lines tested as 60% cell survival in Fig. 4, which indicates that PACA2 cells have some type of resistance against the cytotoxicity of gemcitabine comparing to other two cell lines. For the combination of R3616 with gemcitabine, increased efficacy was observed against all the pancreatic cancer cell lines even if the cells had some resistance to the chemotherapy alone. On the other hand, the combination of

hrR3 with gemcitabine was of weak cytotoxicity toward PACA2 cells, which expressed the most RRM1 by Western blot assay, and the effect was less pronounced than when R3616 was used. These results suggest that, the combination of R3616 and gemcitabine might be suitable for the cancer cell type that is expected to offer resistance to gemcitabine.

In the *in vivo* experiments, the combination of R3616 with gemcitabine yielded a 60% LTS rate (100 days) in the mice. This was higher than in mice treated with an intraperitoneal injection of R3616 alone that resulted in a 50% LTS rate, while mice treated with only hrR3 had a 30% LTS rate; however, there was no statistically significant difference in the LTS rate between R3616 and R3616 with gemcitabine. Thus, combination therapy with R3616 and gemcitabine had the same or slightly higher efficacy than the virus alone. However, mice treated with hrR3 followed by gemcitabine showed a lower LTS rate (20%) than those treated with hrR3 alone. And moreover, there was a statistically significant difference between group A (R3616 + GEM) and group D (hrR3 + GEM) ($P = 0.0078$). From the results of our *in vivo* and *in vitro*, we determined that the

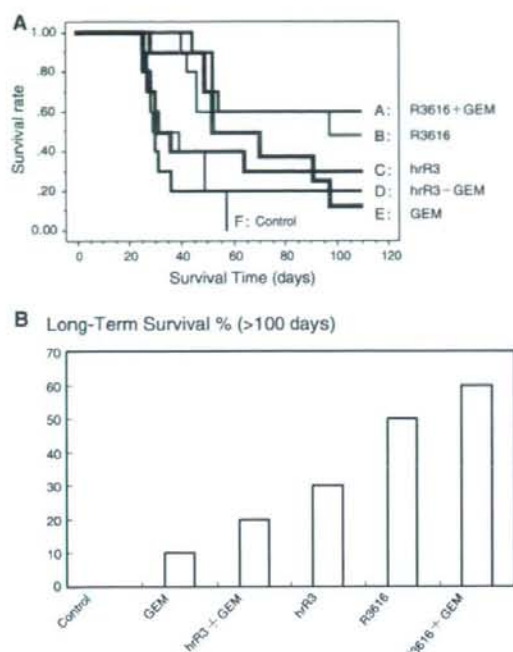


Fig. 6 **a** Cumulative survival curves of an in vivo mouse model. The PACA2 cells were injected into the peritoneal cavity. Each group was treated as shown below. Group A: R3616 + gemcitabine (GEM), group B: R3616 only, group C: hrR3 only, group D: hrR3 + GEM, group E: GEM only, and group F: no treatment. Differences in the survival rates were assessed by log-rank analysis (group A versus group F, $P = 0.0011$; group A versus group D, $P = 0.0078$; group E versus group F, $P = 0.006$; group B versus group D, $P = 0.0174$; and group B versus group F, $P = 0.0049$). There were no other statistically significant differences between the other groups except for shown above. There was a statistically significant difference between group A (R3616 + GEM) and group D (hrR3 + GEM) ($P = 0.0078$). **b** Long-term survival (LTS; over 100 days). LTS was achieved in 60% of mice treated with an intraperitoneal injection of R3616 followed by gemcitabine (group A). Mice treated with an intraperitoneal injection of R3616 only had a 50% LTS survival rate (group B). Mice treated with hrR3 only had a 30% LTS rate (group C). Mice treated with hrR3 followed by gemcitabine had a 20% LTS rate (group D). Mice treated with gemcitabine alone had only a 10% LTS rate (group E). The untreated group (group F) had a 0% LTS survival rate

combination of gemcitabine with R3616 (γ_1 34.5 inactivated) might be more effective than the combination with hrR3 (RR inactivated).

Potentially, chemotherapy drugs connote to inhibit oncolytic virus replication to some degree, but this effect may be influenced by the differences in the characteristics of each virus caused by gene mutation. UL 39 (ICP6)-deleted HSVs, such as G207 [3, 37], and Myb34.5 [38, 39] also have some kind of potential likely to be inhibited by

gemcitabine, as is hrR3, because of the genetic characteristics of RR. In other words, UL39-intact HSVs, such as HF10 [40, 41], RH105 [42], and DF γ 34.5 [43] are likely to interact differently from a UL 39-deleted HSV (e.g., hrR3), in combination therapy with gemcitabine. Additional studies must be needed for further confirmation of the efficacy depending upon the functional characteristics among the chemotherapy drugs, viruses, and the cancer cells.

In the future, oncolytic virus therapy in combination with chemotherapy drugs may become more popular for use in clinical trials. Therefore, the characteristics of each virus must be considered carefully to determine if they are suitable for use with the chemotherapy drugs chosen.

References

- Warshaw AL, Fernandez-del Castillo C (1992) Pancreatic carcinoma. *N Engl J Med* 326:455–464
- Burris HA III, Moore MJ, Andersen J, Green MR, Rothenberg ML, Modiano MR, et al (1997) Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J Clin Oncol* 15:2403–2413
- Kasuya H, Takeda S, Nomoto S, Nakao A (2005) The potential of oncolytic virus therapy for pancreatic cancer. *Cancer Gene Ther* 12(9):725–736
- Germano IM, Fable J, Gultekin SH, Silvers A (2003) Adenovirus/herpes simplex-thymidine kinase/ganciclovir complex: preliminary results of a phase I trial in patients with recurrent malignant gliomas. *J Neuro Oncol* 65:279–289
- Immonen A, Vapalahti M, Tyynela K, Hurskainen H, Sandmair A, Vanninen R, et al (2004) AdvHSV-tk gene therapy with intravenous ganciclovir improves survival in human malignant glioma: a randomized, controlled study. *Mol Ther* 10:967–972
- Teh BS, Ayala G, Aguilar L, Mai WY, Timme TL, Vlachaki MT, et al (2004) Phase I-II trial evaluating combined intensity-modulated radiotherapy and in situ gene therapy with or without hormonal therapy in treatment of prostate cancer: interim report on PSA response and biopsy data. *Int J Radiat Oncol Biol Phys* 58:1520–1529
- Kubo H, Gardner TA, Wada Y, Koeman KS, Gotoh A, Yang L, et al (2003) Phase I dose escalation clinical trial of adenovirus vector carrying osteocalcin promoter-driven herpes simplex virus thymidine kinase in localized and metastatic hormone-refractory prostate cancer. *Hum Gene Ther* 14:227–241
- Mulvihill S, Warren R, Venook A, Adler A, Randlev B, Heise C, et al (2001) Safety and feasibility of injection with an E1B-55 kDa gene-deleted, replication-selective adenovirus (ONYX-015) into primary carcinoma of the pancreas: a phase I trial. *Gene Ther* 8:308–315
- Nakao A, Kimata H, Imai T, Kikumori T, Teshigahara O, Nagasaka T, et al (2004) Intratumoral injection of herpes simplex virus HF10 in recurrent breast cancer. *Ann Oncol* 15:987–991
- Lamont JP, Nemunaitis J, Kuhn JA, Landers SA, McCarty TM (2000) A prospective phase II trial of ONYX-015 adenovirus and chemotherapy in recurrent squamous cell carcinoma of the head and neck (the Baylor experience). *Ann Surg Oncol* 7:588–592
- Neunmaitis J, Cunningham C, Buchnan A, Edelman G, Maples P, Netto G, et al (2001) Intravenous infusion of a replication-selective adenovirus (ONYX-015) in cancer patients: safety, feasibility and biological activity. *Gene Ther* 8:746–759

12. Heinemann V, Hertel LW, Grindey GB, Plunkett W (1988) Comparison of the cellular pharmacokinetics and toxicity of 2',2'-difluoro-deoxycytidine and 1- β -D-arabinofuranosylcytosine. *Cancer Res* 48:4024–4031
13. Huang P, Chubb S, Hertel LW, Plunkett W (1991) Action of 2',2'-difluoro-deoxycytidine on DNA synthesis. *Cancer Res* 51:6110–6117
14. Heinemann V, Xu Y, Chubb S, Sen A, Hertel LW, Grindey GB, et al (1990) Inhibition of ribonucleotide reduction in CCRF-CEM cells by 2', 2'-difluoro-deoxycytidine. *Mol Pharmacol* 38:567–572
15. Rosell R, Felip E, Taron M, Majo J, Mendez P, Sanchez-Ronco M, et al (2004) Gene expression as a predictive marker of outcome in stage (Ib-IIIA-IIIB non-small cell lung cancer after induction gemcitabine-based chemotherapy followed by resectional surgery. *Clin Cancer Res* 10:4215s–4219s
16. Davidson JD, Ma L, Flagella M, Geeganage S, Gelbert LM, Slapak CA (2004) An increase in the expression of ribonucleotide reductase large subunit 1 is associated with gemcitabine resistance in non-small cell lung cancer cell lines. *Cancer Res* 64:3761–3766
17. Rosell R, Danenberg KD, Alberola V, Bepler G, Sanchez JJ, Camps C, et al (2004) Ribonucleotide reductase messenger RNA expression and survival in gemcitabine/cisplatin-treated advanced non-small cell lung cancer patients. *Clin Cancer Res* 10:1318–1325
18. Goan YG, Zhou B, Hu E, Mi S, Yen Y (1999) Overexpression of ribonucleotide reductase as a mechanism of resistance to 2,2'-difluoro-deoxycytidine in the human KB cancer cell line. *Cancer Res* 59:4204–4207
19. Jung CP, Motwani MV, Schwartz GK (2001) Flavopiridol increases sensitization to gemcitabine in human gastrointestinal cancer cell lines and correlates with down-regulation of ribonucleotide reductase M2 subunit. *Clin Cancer Res* 7:2527–2536
20. Duxbury MS, Ito H, Zinner MJ, Ashley SW, Whang EE (2004) RNA interference targeting the M2 subunit of ribonucleotide reductase enhances pancreatic adenocarcinoma chemosensitivity to gemcitabine. *Oncogene* 23:1539–1548
21. Duxbury MS, Ito H, Benoit E, Zinner MJ, Ashley SW, Whang EE (2004) Retrovirally mediated RNA interference targeting the M2 subunit of ribonucleotide reductase: a novel therapeutic strategy in pancreatic cancer. *Surgery* 136:261–269
22. Andreansky SS, He B, Gillespie GY, Soroceanu L, Markert J, Chou J, et al (1996) The application of genetically engineered herpes simplex viruses to the treatment of experimental brain tumors. *Proc Natl Acad Sci USA* 93:11313–11318
23. Pyles RB, Warnick RE, Chalk CL, Szanti BE, Parysek LM (1997) A novel multiply-mutated HSV-1 strain for the treatment of human brain tumors. *Hum Gene Ther* 8:533–544
24. Coukos G, Makrigiannakis A, Kang EH, Rubin SC, Albelda SM, Molnar-Kimber KL (2004) Oncolytic herpes simplex virus-1 lacking ICP34.5 induces p53-independent death and is efficacious against chemotherapy-resistant ovarian cancer. *Clin Cancer Res* 6:3342–3353
25. Kasuya H, Nishiyama Y, Nomoto S, Hosono J, Takeda S, Nakao A (1999) Intraperitoneal delivery of hrR3 and ganciclovir prolongs survival in mice with disseminated pancreatic cancer. *J Surg Oncol* 72:136–141
26. Kasuya H, Mizuno M, Yoshida J, Nishiyama Y, Nomoto S, Nakao A (2000) Combined effects of adeno-associated virus vector and a herpes simplex virus mutant as neoplastic therapy. *J Surg Oncol* 74:214–218
27. Carroll NM, Chiocca EA, Takahashi K, Tanabe KK (1996) Enhancement of gene therapy specificity for diffuse colon carcinoma liver metastases with recombinant herpes simplex virus. *Ann Surg* 224:323–330
28. Kasuya H, Pawlik TM, Mullen JT, Donahue JM, Nakamura H, Chandrasekhar S, et al (2004) Selectivity of an oncolytic herpes simplex virus for cells expressing the DF3/MUC1 antigen. *Cancer Res* 64:2561–2567
29. Yoon SS, Nakamura H, Carroll NM, Bode BP, Chiocca EA, Tanabe KK (2000) An oncolytic herpes simplex virus type 1 selectively destroys diffuse liver metastases from colon carcinoma. *FASEB J* 14:301–311
30. Goldstein DJ, Weller S (1988) Factor(s) present in herpes simplex virus type 1-infected cells can compensate for the loss of the large subunit of the viral ribonucleotide reductase: characterization of an ICP6 deletion mutant. *Virology* 166:41–51
31. Ogg PD, McDonnell PJ, Ryckman BJ, Knudson CM, Roller RJ (2004) The HSV-1 Us3 protein kinase is sufficient to block apoptosis induced by overexpression of a variety of Bcl-2 family members. *Virology* 319:212–224
32. Benetti L, Roizman B (2004) Herpes simplex virus protein kinase Us3 activates and functionally overlaps protein kinase A to block apoptosis. *Proc Natl Acad Sci USA* 101:9411–9416
33. Huang DC, Cory S, Strasser A (1997) Bcl-2, Bcl-XL and adenovirus protein E1B19kD are functionally equivalent in their ability to inhibit cell death. *Oncogene* 14:405–414
34. Teodoro JG, Branton PE (1997) Regulation of p53-dependent apoptosis, transcriptional repression, and cell transformation by phosphorylation of the 55-kilodalton E1B protein of human adenovirus type 5. *J Virol* 71:3620–3627
35. Teodoro JG, Branton PE (1997) Regulation of apoptosis by viral gene products. *J Virol* 71:1739–1746
36. Tschopp J, Thome M, Hoffmann K, Meink E (1998) The fight of viruses against apoptosis. *Curr Opin Genet Dev* 8:82–87
37. Cinatl J Jr, Michaelis M, Driever PH, Cinatl J, Hrabeta J, Suhan T, et al (2004) Multimutated herpes simplex virus g207 is a potent inhibitor of angiogenesis. *Neoplasia* 6:725–735
38. Nakamura H, Kasuya H, Mullen JT, Yoon SS, Pawlik TM, Chandrasekhar S, et al (2002) Regulation of herpes simplex virus g₁ 34.5 expression and oncolysis of diffuse liver metastases by Myb34.5. *J Clin Invest* 109:871–882
39. Chung RY, Saeki Y, Chiocca EA (1999) β -myb promoter retargeting of herpes simplex virus gamma 34.5 gene-mediated virulence toward tumor and cycling cells. *J Virol* 73:7556–7564
40. Teshigahara O, Goshima F, Takao K, Kohno S, Kimata H, Nakao A, et al (2004) Oncolytic viral therapy for breast cancer with herpes simplex virus type 1 mutant HF10. *J Surg Oncol* 85:42–47
41. Kimata H, Takakuwa H, Goshima F, Teshigahara O, Nakao A, Kurata T, et al (2003) Effective treatment of disseminated peritoneal colon cancer with new replication-competent herpes simplex viruses. *Hepatology* 50:961–966
42. Nilaver G, Muldoon LL, Kroll RA, Pagel MA, Breakefield XO, Davidson BL, et al (1995) Delivery of herpesvirus and adenovirus to nude rat intracerebral tumors after osmotic blood-brain barrier disruption. *Proc Natl Acad Sci USA* 92:9829–9833
43. Kasuya H, Pawlik TM, Mullen JT, Donahue JM, Nakamura H, Chandrasekhar S, et al (2004) Selectivity of an oncolytic herpes simplex virus for cells expressing the DF3/MUC1 antigen. *Cancer Res* 64:2561–2567

Pancreatic Cancer With Paraaortic Lymph Node Metastasis A Contraindication for Radical Surgery?

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Objectives: The purpose of this study was to determine the operative indications for pancreatic cancer with paraaortic lymph node metastases (No. 16 [+]).

Methods: Between July 1981 and March 2007, 335 patients with pancreatic cancer including 45 No. 16 (+) patients underwent extended radical surgery at the Department of Surgery II, Nagoya University. The overall survival rates and clinicopathological parameters were analyzed using univariate and multivariate analyses.

Results: Although there was no significant difference in survival between the No. 16 (+) patients and the unresectable cases, there were some long-term survivors among the No. 16 (+) patients. Multivariate analysis of the No. 16 (+) patients identified age (59 years or younger), tumor size (>4 cm), and pathologically confirmed portal invasion (pPV[+]) as independent prognostic factors. The survival of No. 16 (+) patients without these factors was significantly better than the unresectable cases. The survival of patients with only 1 metastatic paraaortic lymph node also was significantly better than the unresectable cases, and tended to be better than those with more than 2 metastatic nodes.

Conclusions: No. 16 (+) pancreatic cancer patients with age 60 years or older, tumor size 4 cm or less, and pPV(-) may benefit from resection.

Key Words: pancreatic cancer, paraaortic lymph node, indication

(*Pancreas* 2009;38: e13-e17)

Pancreatic cancer continues to have the worst prognosis of all the gastrointestinal malignancies, and the actual 5-year survival rate after a curative resection reportedly ranges from 6.8% to 19.8%.¹⁻⁵ Surgical resection remains the only chance for cure, although a moderate improvement in outcome has been achieved through a gradual increase in the resection rate and a decline in the surgical mortality after pancreatoduodenectomy that currently ranges from 1% to 5.4%.¹⁻⁵

Lymph node involvement is one of the most important prognostic factors for gastrointestinal cancer, including pancreatic cancer. Paraaortic lymph nodes (No. 16 nodes) are considered to be the final nodes for periampullary and gastric cancers before the cancer enters the systemic lymphatic circulation. Metastases to the No. 16 nodes (No. 16 [+]) are observed commonly among patients with carcinoma of the head of the pancreas,⁶⁻⁹ and anatomic or clinical studies detailing the

patterns of lymphatic flow from the pancreas to the No. 16 nodes have been reported.^{6,7} Some consider these nodes to be regional lymph nodes and dissect them as a part of a routine lymphadenectomy for pancreatic cancer. Others believe that metastases to these nodes represent systemic disease and recommend that radical surgery including extended lymphadenectomy should be abandoned for No. 16 (+) patients.¹⁰⁻¹² Although the optimal extent of lymphadenectomy for pancreatic cancer thus remains a matter of controversy, there is growing skepticism as to the survival benefit of extended lymphadenectomies in general, and the authors share the opinion that systematic dissection of all No. 16 nodes may not be beneficial when performed routinely for all patients with pancreatic cancer.¹³ On the other hand, we have encountered some No. 16 (+) patients who have experienced long-term survival after an extended nodal resection, suggesting that this procedure may have value for a selected population of patients, the identification of which is the aim of current study.

MATERIALS AND METHODS

Patients Selection and Study Design

Between July 1981 and March 2007, 511 patients with pancreatic cancer underwent surgery at the Department of Surgery II, Nagoya University. Three hundred thirty-five patients had extended radical surgery with systematic lymph node dissection, including regional and No. 16 lymph nodes, whereas 176 patients were deemed unresectable because of macroscopic hepatic metastases, macroscopic peritoneal metastases, or extensive local invasion. The cohort of resected pancreatic cancer patients included 222 men and 113 women, with a median age of 62.2 years (range, 35-83 years). All patients were followed until death or through March 2007. Tumor location included the head of the pancreas (n = 258), the body of the pancreas (n = 68), and the entire pancreas (n = 9). One hundred sixty-one pancreatoduodenectomies, 44 pylorus-preserving pancreatoduodenectomies, 59 distal pancreatectomies, 70 total pancreatectomies, and 1 pancreatic head resection with segmental duodenectomy¹⁴ were performed. The pathologic findings were evaluated in accordance with the second English edition of the *Classification of Pancreatic Carcinoma* proposed by the Japan Pancreatic Society.¹⁵ This classification scheme is more detailed than the classification of the Union Internationale Contre le Cancer.¹⁶ Lymph nodes were classified into several lymph node stations named according to the anatomic location and were numbered: 3, lesser curvature; 4, greater curvature; 5, suprapyloric; 6, infrapyloric; 7, left gastric artery; 8, common hepatic artery; 9, celiac trunk; 10, splenic hilus; 11, splenic artery; 12, hepatoduodenal ligament; 13, posterior pancreatoduodenal; 14, superior mesenteric artery; 15, middle colic artery; 16, paraaortic; 17, anterior pancreatoduodenal; and 18, inferior pancreas lymph nodes. The No. 16 lymph

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TABLE 1. No. Positive and Total Lymph Nodes by Station

Lymph Node Station	Positive LN Number, mean (min-max)	Dissected LN Number, mean (min-max)
No. 3	0.0 (0-0)	0.8 (0-5)
No. 4	0.0 (0-0)	2.4 (0-13)
No. 5	0.0 (0-0)	0.4 (0-3)
No. 6	0.4 (0-4)	2.9 (0-11)
No. 7	0.0 (0-1)	0.7 (0-6)
No. 8	0.4 (0-4)	2.2 (0-10)
No. 9	0.3 (0-11)	1.1 (0-17)
No. 10	0.0 (0-0)	1.0 (0-12)
No. 11	0.2 (0-2)	2.6 (0-21)
No. 12	0.8 (0-4)	4.6 (0-15)
No. 13	1.7 (0-7)	3.7 (0-11)
No. 14	1.9 (0-31)	7.7 (0-38)
No. 15	0.0 (0-0)	0.3 (0-3)
No. 16	2.9 (1-10)	7.4 (1-33)
No. 17	1.5 (0-10)	4.1 (0-13)
No. 18	0.0 (0-1)	0.4 (0-5)
Total	10.1 (1-49)	42.4 (2-105)

nodes in this study refer to those that are surrounded by the celiac trunk, the inferior mesenteric artery, the right margin of the inferior vena cava, and the left margin of the abdominal aorta. Consequently, 45 No. 16 (+) patients were identified. Intraoperative radiation therapy (IORT, 30 Gy) had been administered to the retroperitoneal fields, and 5-fluorouracil (5-FU) portal injection, 5-FU-based chemotherapy, or gemcitabine had been given as postoperative adjuvant chemotherapy to some patients.

Statistical Analysis

The overall survival rates were calculated using the Kaplan-Meier method, and the difference in survival curves was analyzed using the log-rank test. The prognostic value of each clinicopathologic factor was evaluated by univariate analysis among the 45 No. 16 (+) patients. Significant independent prognostic factors were then identified by multivariate analysis using the Cox proportional hazards regression model. Data are expressed as the mean (SD). The level of statistical significance was set at $P < 0.05$.

RESULTS

The numbers of positive and dissected lymph nodes in each of the lymph node stations as defined in the second English edition of the *Classification of Pancreatic Carcinoma*¹⁵ are given in Table 1. The average number of dissected lymph nodes was 42.4 (range, 2-105), and the average number of positive lymph nodes was 10.1 (range, 1-49). As for the No. 16 nodes (No. 16), a mean of 7.4 nodes (range, 1-33 nodes) were dissected, and a metastasis was found in a mean of 2.9 nodes (range, 1-10 nodes).

The overall survival rate of patients stratified by the extent of lymph node involvement is given in Figure 1. Lymph node metastases were observed in 230 (68.7%) of 335 patients. The survival of patients with metastases to the regional nodes (n[+], No. 16 [-]) was significantly worse than that of node-negative patients (n[-]), whereas it was significantly better than that of No. 16 (+) patients ($P = 0.0012$ and $P = 0.0029$, respectively).

On the other hand, there was no significant difference in survival between the No. 16 (+) patients and the unresectable cases.

The clinicopathologic characteristics of the 45 No. 16 (+) patients are provided in Table 2. As postoperative adjuvant chemotherapy, 5-FU portal injection was given to 5 patients, and gemcitabine was injected in 9 patients. Intraoperative radiation therapy was also administered to 26 patients. The survival time of the No. 16 (+) subjects ranged from 0.1 to 45.4 months (median, 7.8 months). There were some long-term survivors even among this population.

Univariate analysis among the No. 16 (+) patients revealed that age 59 years or younger, tumor size greater than 4 cm, pathological portal vein invasion (pPV[+]), and perineural invasion were the factors significantly associated with survival (Table 3). These 4 variables were included in the multivariate analysis using Cox proportional hazards regression model along with clinical portal vein invasion, a factor that was almost significant in the univariate analysis. Consequently, age 59 years or younger, tumor size greater than 4 cm, and pPV(+) were identified as independent prognostic factors among this population (Table 4). The overall survival curves stratified for these 3 independent prognostic factors are shown in Figures 2A-C.

Finally, the survival of the No. 16 (+) cases was evaluated. These cases were subclassified into 2 groups, patients with only 1 metastatic No. 16 lymph node and those with 2 or more nodes. The survival of patients with 1 positive lymph node was significantly better than that of the unresectable cases and tended to be better than that of patients with 2 or more positive nodes ($P = 0.049$ and $P = 0.14$, respectively; Fig. 2D).

DISCUSSION

Lymph nodes are the most frequent site of metastases for gastrointestinal cancer, including pancreatic cancer, and their removal in theory offers a therapeutic potential, particularly to achieve local control. Efforts to obtain improved tumor clearance through enlarging the anatomic extent of lymphadenectomy have often been found unrewarding in survival benefit not only for pancreatic cancer¹⁷⁻²⁰ but also for gastric

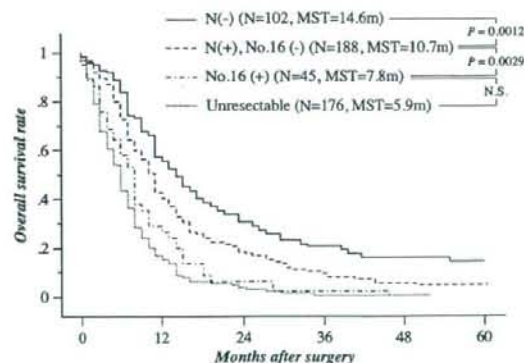


FIGURE 1. The overall survival rates of patients based on lymph node involvement (n) are shown. The survival of patients with n(+), No. 16 (-) was significantly worse compared with those who were n(-) and was significantly better than those with No. 16 (+) ($P = 0.0012$ and $P = 0.0029$, respectively). There was no significant difference in the survival curves of the patients with the No. 16 (+) and the unresectable cases. m indicates months; MST, mean survival time; NS, not statistically significant.

TABLE 2. Patient Characteristics With Paraortic Lymph Node Metastases

Age, range (mean [SD]), y	43–79 (62.5 [7.8])
Sex	
Male	29
Female	16
Tumor location	
Head	39
Body, tail	5
Whole	1
Operative procedure	
PD	24
PpPD	2
DP	4
TP	15
Postoperative chemotherapy	
5-FU	5
Gemzal	9
Others	3
None	26
IORT	
30 Gy	26
None	19
Survival time, range (mean [SD]), mo	0.1–45.4 (9.6 [8.6])

DP indicates distal pancreatectomy; PD, pancreatoduodenectomy; PpPD, pylorus-preserving pancreatoduodenectomy; TP, total pancreatectomy.

cancer.^{21–23} These findings and experience with other types of cancer suggest that the indications for extended lymphadenectomy should be seriously reconsidered.

There is a general consensus, however, that localized pancreatic cancer with regional lymph node metastases can be cured only by extended radical surgery. According to recent clinical observations, the absolute contraindications for pancreatectomy are the presence of liver metastases and peritoneal deposits, whereas the relative contraindications are the involvement of the portal venous system or major arteries. In addition, some authors suggest that radical surgery with extended lymphadenectomy should be abandoned for No. 16 (+) patients because long-term survival is extremely infrequent in this population, although extended lymphadenectomy did not adversely affect morbidity or mortality.^{10–12} Consequently, radical surgery may not be recommended when a No. 16 (+) lymph node is confirmed during surgery through sampling and intraoperative examination of the frozen section. On the other hand, the inclusion of No. 16 to the routine range of lymphadenectomy, although time-consuming, is technically feasible, with acceptable morbidity and mortality reported from the authors and from others.^{13,17,19} Furthermore, there were some patients with paraortic lymph node involvement who had a chance of long-term survival.^{6–9} Therefore, it is important to identify No. 16 (+) patients who may benefit from radical surgery with extended lymphadenectomy.

The purpose of this study was to determine the indications for the resection of No. 16 (+) pancreatic cancer. The survival of No. 16 (+) patients was significantly worse than those with n(+) No. 16 (–) disease, whereas no significant difference in survival was observed between the No. 16 (+) patients and the unresectable cases in this study. Given these results, No. 16 (+) was

initially considered not to be a good target for radical surgery. The results in the current study indicate that age 59 years or younger, tumor size 4 cm or greater, and pPV(+) are independent factors that predict a poor outcome after radical surgery for No. 16 (+) patients, and the survival of patients without these factors was significantly better than that of patients who were deemed unresectable. Because the tumor of No. 16 (+) patients was mainly located in the head of pancreas in this study, we could not evaluate the prognostic relation in cancer location or operative procedure and could not deny biologic involvement. Along with size of the tumor, age has been reported to affect prognosis in some other cancer types. Early age of onset is often considered a poor prognostic factor for colorectal cancer, for example, which tends to be diagnosed at a more advanced stage. It tends to show more aggressive histopathologic features and to result in lower survival rates in younger patients.^{24,25} On the other hand, pPV(+) may not be as useful for making a decision regarding whether or not to proceed with radical surgery, because this information is essentially unavailable before surgical resection and histopathologic examination. However, the authors have shown that intraportal endovascular ultrasonography (IPEUS) is capable of accurately detecting or excluding histologic invasion of the portal vein wall.^{26,27} Correlation of IPEUS results with pathologic examination of resected specimens revealed that tumor-vessel contiguity with an intact echogenic band was indicative of tumor within 1 mm of the adventitia of the portal vein wall but without actual invasion. Although most centers might not use this IPEUS, recent intraoperative ultrasonography (IOUS), which provides a distinct image, could be used as a substitute for IPEUS. It can be assumed, therefore, that future indications for radical surgery in No. 16 (+) patients could be decided based on the age, tumor size, and the IPEUS or IOUS findings.

Recent reports indicated that the number of positive nodes at a given lymph node station is an important predictor for survival of the surgically treated patients.²⁸ It was also reported

TABLE 3. Univariate Analysis for Patients With Paraortic Lymph Node Metastases

Variable	Odds Ratio	95% CI	P
Age (≤ 59 y)	2.354	1.226–4.520	0.0101
Sex (male)	0.981	0.524–1.836	0.9517
Location			
Body, tail	1.008	0.136–7.459	0.9937
Whole	1.217	0.475–3.118	0.6825
Tumor size (>4 cm)	3.589	1.789–7.200	0.0003
PV(+)	2.652	0.909–7.738	0.0742
Vascular invasion	1.177	0.577–2.401	0.6547
Invasion of anterior pancreatic capsule	1.346	0.737–2.460	0.3335
Invasion of retroperitoneal tissue	1.803	0.880–3.693	0.1073
Bile duct invasion	0.778	0.370–1.636	0.5086
Duodenal invasion	1.326	0.706–2.490	0.3804
pPV(+)	2.051	1.033–4.073	0.0401
Arterial invasion	1.716	0.851–3.461	0.1315
Perineural invasion	2.045	1.008–4.148	0.0474
pDPM	1.641	0.881–3.056	0.1184
No. 16–positive (≥ 2)	1.585	0.851–2.952	0.1464

CI indicates confidence interval; PV, clinical portal vein invasion; pDPM, pathological dissected peripancreatic tissue margin.

TABLE 4. Multivariate Analysis for Patients with Paraortic Lymph Node Metastases

Variable	Odds Ratio	95% CI	P
Age (≤ 59 years)	3.438	1.621–7.290	0.0013
Tumor size (>4 cm)	2.693	1.304–5.561	0.0074
PV(+)	1.062	0.304–3.717	0.9245
pPV(+)	2.359	1.055–5.278	0.0367
Perineural invasion	1.84	0.868–3.898	0.1115

that the removal and pathologic examination of a greater number of lymph nodes can influence staging accuracy and even improve the overall survival after pancreatotomy.²⁹ Furthermore, some authors revealed that the ratio of the number of positive lymph nodes to the total number of dissected lymph nodes (lymph node ratio) was one of the most powerful predictors of survival.^{30,31} In this study, the mean number of lymph nodes retrieved was 42.4, and the mean number of metastatic lymph nodes was 10.1. These data are comparable to what has been reported in the literature. The survival of patients with only 1 metastatic No. 16 lymph node was significantly better than the unresectable cases and also tended to be better than those with more than 2 metastatic nodes, although the difference did not

reach statistical significance due to the small sample size. Regarding the relationship between No. 16 (+) and other stations, as we reported previously, statistical analysis showed that metastases to paraortic lymph nodes had a strong correlation with metastases to Nos. 12, 13, 14, and 17 lymph nodes, and they were seldom observed among the patients who had no metastases to Nos. 13, 14, and 17 lymph nodes.⁷ Before performing IPEUS or IOUS, therefore, sampling of the paraortic lymph nodes and subsequent evaluation of the frozen sections could facilitate a decision regarding whether the patient had only 1 positive paraortic lymph node. Our data might indicate that radical surgery should be abandoned when 2 or more examined lymph nodes harbor metastases.

Finally, the effect of adjuvant therapies need to be mentioned, because the treatment strategy of the patients analyzed in this study was rather mixed. No significant difference in survival was observed between the patients who received chemotherapy and those who did not. Likewise, IORT performed in 26 patients did not confer any survival benefit (data not shown). The difference in adjuvant therapies given to the patients can therefore be considered to have had little influence on the results obtained in this study. This lack of efficacy of adjuvant treatment indeed justifies our policy to find ways to increase candidates for radical surgery, the only treatment modality that has been shown to have a significant impact on the survival of patients.

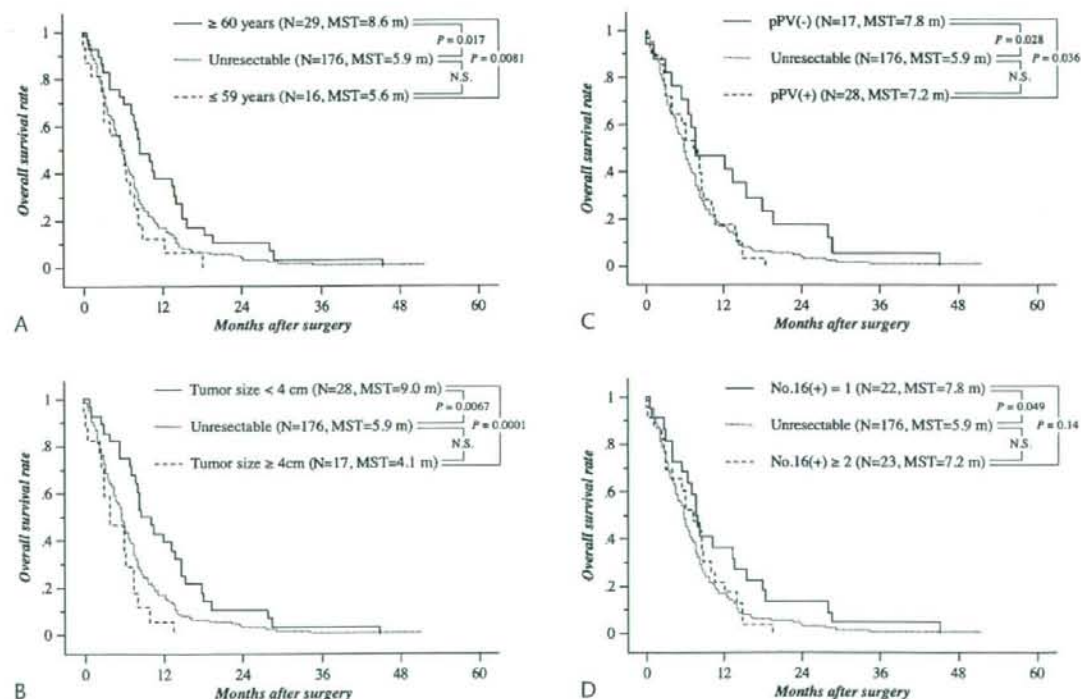


FIGURE 2. A, The overall survival curves based upon patient age. The survival of patients 60 years or older was significantly better than those 59 years or younger and those who were unresectable ($P = 0.081$ and $P = 0.017$, respectively). B, The overall survival curves were based on tumor size. The survival of patients with tumors less than 4 cm was significantly better than those with tumors 4 cm or greater and those who were unresectable ($P = 0.0001$ and $P = 0.0067$, respectively). C, The overall survival curves based upon pPV. The survival of patients with pPV(-) disease was significantly better than those who were pPV(+) or were unresectable ($P = 0.036$ and $P = 0.028$, respectively). D, The survival of patients with 1 positive lymph node was significantly better than those who were unresectable and tended to have a better survival than patients with 2 or more positive lymph nodes ($P = 0.049$ and $P = 0.14$, respectively).

In this retrospective analysis, there were 3 No. 16 (+) patients who survived for more than 2 years, all of whom were 60 years or older, had a tumor size of 4 cm or less, were pPV(-), and had only 1 metastatic paraaortic lymph node. These factors indicate that these are relatively promising targets for radical surgery among the No. 16 (+) population who usually have a dismal prognosis. Although these findings, along with the accuracy of IPEUS or IOUS in identifying pPV(-) patients, will have to be confirmed by a prospective study, radical resection with extended lymphadenectomy remains an option for selected No. 16 (+) patients at this time.

REFERENCES

- Nitecki SS, Sarr MG, Colby TV, et al. Long-term survival after resection for ductal adenocarcinoma of the pancreas. Is it really improving? *Ann Surg.* 1995;221:59-66.
- Conlon KC, Klimstra DS, Brennan MF. Long-term survival after curative resection for pancreatic ductal adenocarcinoma. Clinicopathologic analysis of 5-year survivors. *Ann Surg.* 1996;223:273-279.
- Cameron JL, Riall TS, Coleman J, et al. One thousand consecutive pancreaticoduodenectomies. *Ann Surg.* 2006;244:10-15.
- Capussotti L, Massucco P, Ribero D, et al. Extended lymphadenectomy and vein resection for pancreatic head cancer: outcomes and implications for therapy. *Arch Surg.* 2003;138:1316-1322.
- Wagner M, Reddaelli C, Lietz M, et al. Curative resection is the single most important factor determining outcome in patients with pancreatic adenocarcinoma. *Br J Surg.* 2004;91:586-594.
- Kayahara M, Nagakawa T, Ohta T, et al. Analysis of paraaortic lymph node involvement in pancreatic carcinoma. *Cancer.* 1999;85:583-590.
- Sakai M, Nakao A, Kaneko T, et al. Para-aortic lymph node metastasis in carcinoma of the head of the pancreas. *Surgery.* 2005;137:606-611.
- Ishikawa O, Ohhigashi H, Sasaki Y, et al. Practical usefulness of lymphatic and connective tissue clearance for the carcinoma of the pancreas head. *Ann Surg.* 1988;208:215-220.
- Nakao A, Harada A, Nonami T, et al. Lymph node metastasis in carcinoma of the head of the pancreas region. *Br J Surg.* 1995;82:390-402.
- Yoshida T, Matsumoto T, Sasaki A, et al. Outcome of paraaortic node-positive pancreatic head and bile duct adenocarcinoma. *Am J Surg.* 2004;187:736-740.
- Shimada K, Sakamoto Y, Sano T, et al. The role of paraaortic lymph node involvement on early recurrence and survival after macroscopic curative resection with extended lymphadenectomy for pancreatic carcinoma. *J Am Coll Surg.* 2006;203:345-352.
- Doi R, Kami K, Ito D, et al. Prognostic implication of para-aorta lymph node metastasis in resectable pancreatic cancer. *World J Surg.* 2007;31:147-154.
- Nakao A, Takeda S, Inoue S, et al. Indications and techniques of extended resection for pancreatic cancer. *World J Surg.* 2006;30:976-982.
- Nakao A. Pancreatic head resection with segmental duodenectomy and preservation of gastroduodenal artery. *Hepatogastroenterology.* 1998;45:533-535.
- Japan Pancreatic Society. *Classification of Pancreatic Carcinoma.* 2nd English ed. Tokyo, Japan: Kanehara Pub; 2003.
- Sobin LH, Wittekind CH. *TNM Classification of Malignant Tumours.* 6th ed. New York, NY: Wiley-Liss; 2002.
- Pedrazzoli S, DiCarlo V, Dionigi R, et al. Standard versus extended lymphadenectomy associated with pancreatoduodenectomy in the surgical treatment of adenocarcinoma of the head of the pancreas. *Ann Surg.* 1998;228:508-514.
- Henne-Bruns D, Vogel I, Lutjens J, et al. Surgery for ductal adenocarcinoma of the pancreatic head: staging, complications, and survival after regional versus extended lymphadenectomy. *World J Surg.* 2000;24:595-602.
- Yeo CJ, Cameron JL, Lillemore KD, et al. Pancreaticoduodenectomy with or without distal gastrectomy and extended retroperitoneal lymphadenectomy for periampullary adenocarcinoma, part 2: randomized controlled trial evaluating survival, morbidity, and mortality. *Ann Surg.* 2002;236:355-366.
- Farnell MB, Pearson RK, Sarr MG, et al. A prospective randomized trial comparing standard pancreatoduodenectomy with pancreatoduodenectomy with extended lymphadenectomy in resectable pancreatic head adenocarcinoma. *Surgery.* 2005;138:618-630.
- Robertson CS, Chung SCS, Woods SDS, et al. A prospective randomized trial comparing R₁ subtotal gastrectomy with R₃ total gastrectomy for antral cancer. *Ann Surg.* 1994;200:176-182.
- Cuschieri A, Weeden S, Fielding J, et al. Patients survival after D₁ and D₂ resections for gastric cancer: long-term results of the MRC randomized surgical trial. *Br J Surg.* 1999;79:1522-1530.
- Hartgrink HH, van de Velde CJH, Putter H, et al. Extended lymph node dissection for gastric cancer: who may benefit? Final results of the randomized Dutch Gastric Cancer Group Trial. *J Clin Oncol.* 2004;22:2069-2077.
- Cusack JC, Giacco GG, Cleary K, et al. Survival factors in 186 patients younger than 40 years old with colorectal adenocarcinoma. *J Am Coll Surg.* 1996;183:105-112.
- Quah HM, Joseph R, Schrag D, et al. Young age influences treatment but not outcome of colon cancer. *Ann Surg Oncol.* 2007;14:2759-2765.
- Kaneko T, Nakao A, Inoue S, et al. Intraportal endovascular ultrasonography in the diagnosis of portal vein invasion by pancreatobiliary carcinoma. *Ann Surg.* 1995;222:711-718.
- Nakao A, Kaneko T. Intravascular ultrasonography for assessment of portal vein invasion by pancreatic carcinoma. *World J Surg.* 1999;23:892-895.
- Brennan MF, Kattan MW, Klimstra D, et al. Prognostic nomogram for patients undergoing resection for adenocarcinoma of the pancreas. *Ann Surg.* 2004;240:293-298.
- Schwarz RE, Smith DD. Extent of lymph node retrieval and pancreatic cancer survival: Information from a large US population database. *Ann Surg Oncol.* 13:1189-1200.
- Berger AC, Watson JC, Ross EA, et al. The metastatic/examined lymph node ratio is an important prognostic factor after pancreatoduodenectomy for pancreatic adenocarcinoma. *Am Surg.* 2004;70:235-240.
- Pawlik TM, Gleisner AL, Cameron JL, et al. Prognostic relevance of lymph node ratio following pancreatoduodenectomy for pancreatic cancer. *Surgery.* 2007;141:610-618.

A New Technique for Intraoperative Continuous Biliary Drainage during Pancreatoduodenectomy

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Key Words

Pancreaticoduodenectomy · Intraoperative biliary drainage · Preoperative biliary drainage

Abstract

Background: The common hepatic duct is divided during the early stage of pancreatoduodenectomy. Complete and prolonged closure of the proximal common duct stump can cause liver damage in the course of this long operation, resulting in associated complications. **Methods:** We performed intraoperative continuous external bile drainage by a new method using a novel drainage clamp in 47 consecutive patients (drainage clamp group) and compared postoperative liver enzyme levels, inflammation markers, morbidity, and outcomes with those of a conventional clamp group (n = 40). **Results:** The drainage clamp group had significantly lower transaminase levels within the first 14 postoperative days than the conventional clamp group. The number of patients with elevated transaminase was significantly less in drainage clamp group than conventional clamp group ($p < 0.001$). There were no significant differences between these two groups in terms of mortality rates and postoperative morbidity. **Conclusion:** Intraoperative complete closure of the common hepatic duct contributed to postoperative elevated transaminase levels, and the continuous decompression of the hepatic duct during pancreatoduodenectomy is beneficial to patients by avoiding liver dysfunction. The novel

drainage clamp is a safe and useful tool for pancreatoduodenectomy and other operative procedure where extrahepatic bile duct is dissected. Copyright © 2008 S. Karger AG, Basel

Introduction

In most standard pancreaticoduodenectomy and pylorus-preserving pancreaticoduodenectomy procedures, the common hepatic duct is divided during the early stage of the operative procedure [1]. The proximal common duct stump is usually closed with an atraumatic bulldog vessel clamp to prevent intraoperative peritoneal bile contamination. The bile duct clamp causes abrupt and complete biliary obstruction lasting for several hours before completion of hepaticojejunal anastomosis. The clamp is released periodically, and hepatic bile juice is flushed out and sucked away, although even this maneuver involves a chance of peritoneal bile contamination. In addition, prolonged proximal clamp of the common hepatic duct can cause elevated biliary pressure and biliary reflux through hepatic sinusoid that may result in a hepatic cell damage, consequent postoperative liver dysfunction, hyperbilirubinemia, or liver abscess. Therefore, to reduce intraoperative liver damage, continuous drainage of bile after common hepatic duct closure is desirable.

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When a percutaneous transhepatic biliary drainage (PTBD) catheter is placed before surgery, it can serve as an intraoperative drainage route. In these patients, the complete biliary obstruction by vessel clamp does not increase biliary pressure, and we noted that no serious elevation of postoperative hepatic transaminase data. However, patients who are not jaundiced undergo pancreaticoduodenectomy without biliary drainage. In addition, it has been reported that many centers perform surgery without biliary drainage even in jaundiced patients [2, 3]. Therefore, we thought that intraoperative continuous bile drainage by placing a catheter immediately after dividing the common hepatic duct would better work for preventing the elevation of biliary pressure and hepatic damage.

For this purpose, we have developed a novel clamp with a shape that can easily fix a drainage catheter. To determine the effectiveness and necessity of intraoperative continuous bile drainage, we retrospectively compared the postoperative course and liver function test results of these intraoperative continuous bile drainage patients with those of an historical control group without intraoperative continuous bile drainage.

Materials and Methods

Patients

Patients who underwent pancreaticoduodenectomy in Kyoto University Hospital between January 2004 and December 2006 had been enrolled in this study. Patients who had had PTBD were excluded, because intraoperative biliary decompression is attained by PTBD in these patients. Patients with preoperative abnormal liver function, defined by aspartate aminotransferase (AST) >200 IU/l, alanine aminotransferase (ALT) >200 IU/l, or total bilirubin >5.0 mg/dl, and patients with perioperatively diagnosed liver cirrhosis and liver metastasis were also excluded from this study because these conditions affect the evaluation of intraoperative biliary decompression.

Technique of Pancreaticoduodenectomy

All patients underwent preoperative bowel preparation with a polyelectrolyte solution, and received perioperative intravenous antibiotic prophylaxis using a second-generation cephalosporin starting 1 h before skin incision. All patients underwent pancreaticoduodenectomy or pylorus-preserving pancreaticoduodenectomy. The same two senior surgeons performed all the operations in this series. When common hepatic duct was transected, a conventional non-crushing curved bulldog vein clamp (Mizuho Co., Ltd., Tokyo, Japan) or a newly developed curved drainage clamp (Mizuho Co., Ltd.) was positioned across the hepatic duct. When a conventional clamp was used, an intermittent opening of the bile duct was repeated periodically to minimize an elevation of biliary pressure during the period between division of the common hepatic duct and subsequent biliary-enteric reconstruction. Figure 1 shows the newly developed drainage clamp that has a

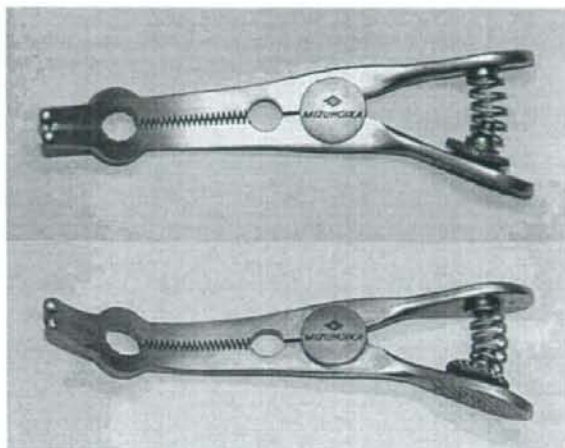


Fig. 1. The novel drainage clamp. The clamp has a hole with a diameter of 6 mm through which a 12- or 14-Fr catheter can be placed.

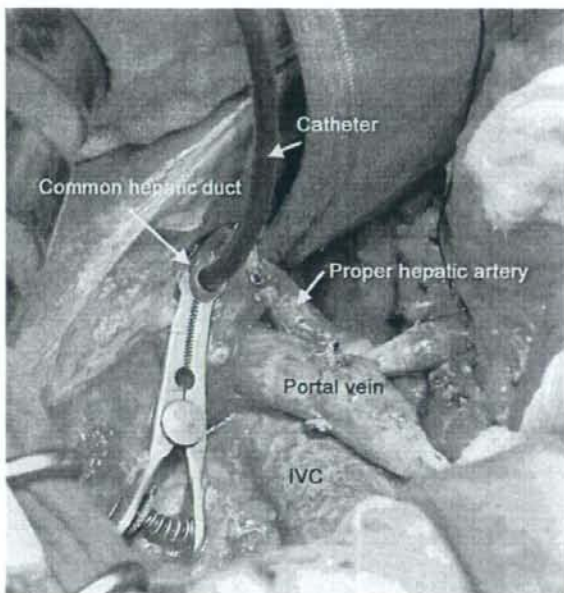


Fig. 2. Intraoperative procedure of continuous bile drainage by a drainage clamp and a nelaton catheter. A nelaton catheter was introduced into the common hepatic duct stump, and a drainage clamp was placed on it. The catheter was connected to an extracorporeal tube.