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LIM only 4 is overexpressed in late stage pancreas cancer

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Abstract

Background: LIM-only 4 (LMO4), a member of the LIM-only (LMO) subfamily of LIM domain-containing transcription factors, was initially reported to have an oncogenic role in breast cancer. We hypothesized that LMO4 may be related to pancreatic carcinogenesis as it is in breast carcinogenesis. If so, this could result in a better understanding of tumorigenesis in pancreatic cancer.

Methods: We measured *LMO4* mRNA levels in cultured cells, pancreatic bulk tissues and microdissected target cells (normal ductal cells; pancreatic intraepithelial neoplasia-1B [PanIN-1B] cells; PanIN-2 cells; invasive ductal carcinoma [IDC] cells; intraductal papillary-mucinous adenoma [IPMA] cells; IPM borderline [IPMB] cells; and invasive and non-invasive IPM carcinoma [IPMC]) by quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR).

Results: 9 of 14 pancreatic cancer cell lines expressed higher levels of *LMO4* mRNA than did the human pancreatic ductal epithelial cell line (HPDE). In bulk tissue samples, expression of *LMO4* was higher in pancreatic carcinoma than in intraductal papillary-mucinous neoplasm (IPMN) or non-neoplastic pancreas ($p < 0.0001$ for both). We carried out microdissection-based analyses. IDC cells expressed significantly higher levels of *LMO4* than did normal ductal epithelia or PanIN-1B cells ($p < 0.001$ for both) or PanIN-2 cells ($p = 0.014$). IPMC cells expressed significantly higher levels of *LMO4* than did normal ductal epithelia ($p < 0.001$), IPMA ($p < 0.001$) and IPMB cells ($p = 0.003$).

Conclusion: Pancreatic carcinomas (both IDC and IPMC) expressed significantly higher levels of *LMO4* mRNA than did normal ductal epithelia, PanIN-1B, PanIN-2, IPMA and IPMB. These results suggested that *LMO4* is overexpressed at late stages in carcinogenesis of pancreatic cancer.

Introduction

Pancreatic cancer is one of the most aggressive malignant tumors. It is the fifth leading cause of cancer death in Japan [1,2] and has the lowest survival rate of any solid cancer [3]. Because the lack of specific symptoms in patients with pancreatic cancer makes early diagnosis difficult, initial diagnosis typically occurs when the tumor has reached an advanced stage [4]. A better understanding of pancreatic carcinogenesis is urgently needed to facilitate early detection. Pancreatic intraepithelial neoplasia (PanIN) and intraductal papillary-mucinous neoplasm (IPMN) were reported to be precursor lesions of pancreatic cancer [5-8]. Development of invasive ductal adenocarcinoma has been proposed to occur via two pathways [9-11], the PanIN-Invasive ductal carcinoma (IDC) progression pathway and the IPM adenoma (IPMA)-invasive IPM carcinoma (IPMC) pathway, although some specific subtypes of IPMN, such as intestinal-type IPMN, may not progress to invasive carcinoma through the same genetic pathway as PanIN. Longnecker *et al* [12] reported that PanIN-1 and IPMA showed mild dysplasia (grade 1), PanIN-2 and IPM borderline (IPMB) lesions showed moderate dysplasia (grade 2), and PanIN-3 and IPMC (carcinoma *in situ* [CIS]) showed severe dysplasia (grade 3).

LIM-only 4 (LMO4) is one of the four members (LMOs 1, 2, 3 and 4) of the LIM-only subfamily of LIM domain proteins. LIM domains are an approximately 55-amino acid, cysteine-rich, zinc-binding motif that mediate protein-protein interactions present in a variety of proteins including LIM homeobox proteins [13]. The nuclear LIM-only proteins (LMOs 1–4) lack a DNA-binding domain but still function as transcriptional regulators by recruiting other protein partners including transcription factors [14,15]. Kenny *et al* [13] reported the isolation and characterization of *LMO4*, a novel LIM-only gene that is highly expressed in the T-lymphocyte lineage, cranial neural crest cells, somites, dorsal limb bud mesenchyme, motor neurons and Schwann cell progenitors. As well as its role in development, there are several lines of evidence suggesting that LMO4 may have roles in oncogenesis [16]. LMO4, initially described as a human breast tumor autoantigen [17], was reported to have a role in maintaining proliferation of mammary epithelium and suggested that deregulation of this gene may contribute to breast tumorigenesis [18]. Additionally, Sum *et al* [19] found that LMO4 interacts with the cofactor CtIP and the tumor suppressor breast cancer 1 (BRCA1), and inhibits the transcriptional activity of BRCA1 in both yeast and mammalian cells by functional assays. They concluded that deregulation of LMO4 in breast epithelium directly contributes to breast neoplasia by altering the rate of cellular proliferation and promoting cell invasion. In 2005, Sum and colleagues reported that *LMO4* mRNA was overexpressed in 5 of 10 human breast cancer cell lines; *in situ* hybridization analysis of 177 primary invasive breast carcinomas revealed overexpression of *LMO4* in 56% of the specimens [20]. It has also been reported that expression of *LMO4* is up-regulated at the invasive front of oral cancer, suggesting a role in cancer cell invasion [21]. It was recently reported that the bone morphogenic protein (*BMP7*) gene, which controls cell proliferation and apoptosis of mammary epithelial cells, is a direct target of *LMO4* [22]. Both pancreatic cancer and breast cancer are known to have an epithelial origin while pancreatic cancer also reveals papillo-tubular structures that are similar to the histological characteristics of the initial breast cancer [23]. As well, known genetic changes in pancreatic cancer often involve the same genes as those found in breast cancer [24]. Taken together, these findings suggest that LMO4 has critical functions in pancreatic carcinogenesis as well as in normal development. Thus clarification of the role of LMO4 may be useful for diagnosis and/or treatment of pancreatic cancer. However, little is known about the role of *LMO4* in pancreatic cancer and carcinogenesis.

To determine whether LMO4 is correlated with pancreatic cancer and carcinogenesis, we quantified *LMO4* mRNA levels in cultured pancreatic cell lines, bulk tissues and microdissection-based target cells (including normal pancreatic ductal, PanIN-1B and PanIN-2, IDC, IPMA, IPMB and IPMC cells), by quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR). Our goal was to characterize *LMO4* expression in the early and late stages of pancreatic carcinogenesis to clarify both if and when overexpression of *LMO4* occurs.

Materials and methods

Cultured cells

Fourteen pancreatic cancer cell lines, AsPC-1, KP-1N, KP-2, KP-3, PANC-1, SUIT-2 (provided by Dr. H. Iguchi, National Shikoku Cancer Center, Matsuyama, Japan), MIA PaCa-2 (Japanese Cancer Resource Bank, Tokyo, Japan), NOR-P1 (established in our laboratory), CAPAN-1, CAPAN-2, CFPAC-1, H48N, HS766T and SW1990 (American Type Culture Collection, Manassas, VA, USA), the HPDE cell line and six primary cultures of fibroblasts derived from pancreatic tumors were studied. Cells were maintained as described previously [25].

Pancreatic tissues

Tissue samples were obtained during surgery at Kyushu University Hospital (Fukuoka, Japan) as described previously [26]. In brief, tissue samples were removed and divided into at least three bulk tissue specimens. The first sample was immediately but temporarily preserved in cold PBS and then embedded in OCT compound (Sakura Findek, Tokyo, Japan), snap-frozen for microdissection within 1 hour after resection, and stored at -80°C until

use. The second sample was fixed in formalin, embedded in paraffin and cut into 4-μm-thick sections for hematoxylin and eosin (H&E) staining. The third sample was immediately snap-frozen for bulk tissue analysis and stored at -80°C until use. Tissues adjacent to the specimens were examined histologically and the diagnosis confirmed by two pathologists, Dr Hiroshi Yamaguchi and Dr Kohei Nakata (Figure 1). Written informed consent was obtained from all patients, and the study was approved by our institution's surveillance committee and conducted according to the Helsinki Declaration.

RNA isolation

Total RNA was extracted from cultured cells with a High Pure RNA Isolation Kit (Roche, Mannheim, Germany). Total RNA was extracted from bulk tissues with an RNeasy Mini Kit (Qiagen, Tokyo, Japan) following the manufacturer's protocol. Total RNA was extracted from cells isolated by microdissection with the standard acid guanidinium thiocyanate-phenol-chloroform protocol [27] with or without glycogen (Funakoshi, Tokyo, Japan) [26].

Quantitative assessment of LMO4 *level by real-time RT-PCR*

Quantitative real-time RT-PCR was performed with a QuantiTect SYBR Green RT-PCR Kit (Qiagen, Tokyo, Japan) with a Chromo4™ System (Bio-Rad, Hercules, CA, USA). In brief, the reaction mixture was first incubated at 50°C for 30 min to allow for reverse transcription. PCR was initiated with one cycle of 95°C for 15 min to activate the modified Taq polymerase followed by 40 cycles of 94°C for 15 sec, 55°C for 20 sec, and 72°C for 10 sec, and one cycle of 95°C for 0 sec, 65°C for 15 sec and +0.1°C/sec to 95°C for melting analysis. Each sample was run in triplicate. The level of *LMO4* mRNA expression was calculated from a standard curve constructed with total RNA from the SUIT-2 pancreatic cancer cell line. The range of threshold cycles was from 20–35 cycles for *LMO4* primers (forward, 5'-GGA CCG CTT TCT GCT CTA TG-3'; reverse, 5'-AAG GAT CAT GCC ACT TTT GG-3'), and from 7–35 cycles for *18S* rRNA primers (forward 5'-GAT ATG CTC ATG TGG TGT TG-3'; reverse, 5'-AAT CTT CTT CAG TCG CTC CA-3'). Expressions of *LMO4* mRNA were normalized to that of *18S* rRNA.

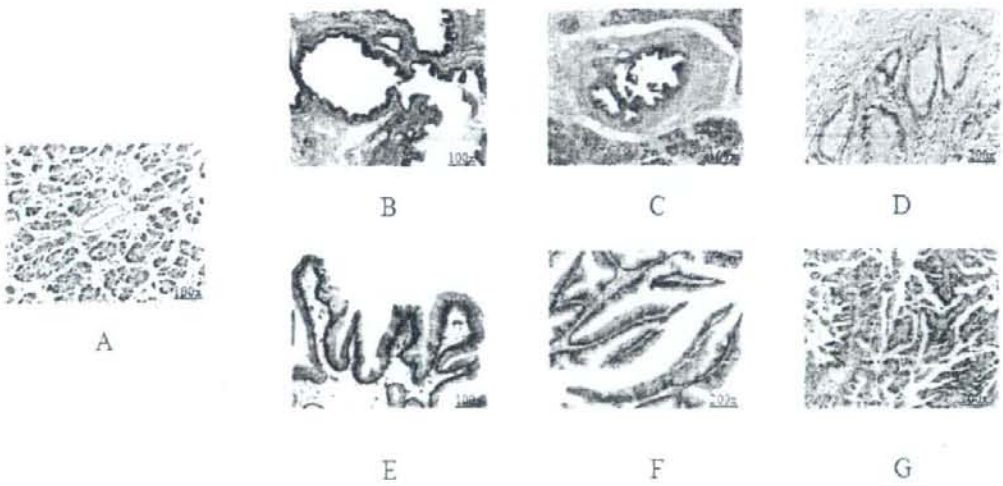

Figure 1

Histologic examination of formalin-fixed, paraffin-embedded (FFPE) samples with hematoxylin and eosin (H&E) staining. The stages of the PanIN-IDC pathway are shown as normal pancreatic ductal epithelia (A), pancreatic intraepithelial neoplasm-1B (PanIN-1B) (B), PanIN-2 (C) and invasive ductal carcinoma (IDC) (D). The stages of the intraductal papillary mucinous adenoma (IPMA)-intraductal papillary mucinous carcinoma (IPMC) pathway are shown as IPM adenoma (E), IPM borderline (F) and IPM carcinoma (G). (Magnifications of A to G are 100×, 100×, 100×, 200×, 100×, 200×, 200×, respectively).

Microdissection-based quantitative analysis of LMO4 *mRNA*

Frozen tissues were cut into 8-μm-thick sections. IDC cells from 8 lesions, PanIN-2 cells from 3 lesions, PanIN-1B cells from 14 lesions, normal ductal epithelial cells from 13 ducts, IPMA cells from 13 lesions, IPMB cells from 15 lesions, and IPMC cells from 8 lesions, including 3 non-invasive IPMC lesions (CIS) and 5 invasive IPMC lesions, were selectively isolated with a laser microdissection and pressure catapulting system (P.A.L.M. Microlaser Technologies, Bernried, Germany) in accordance with the manufacturer's protocols (Figure 2) [28]. After microdissection,

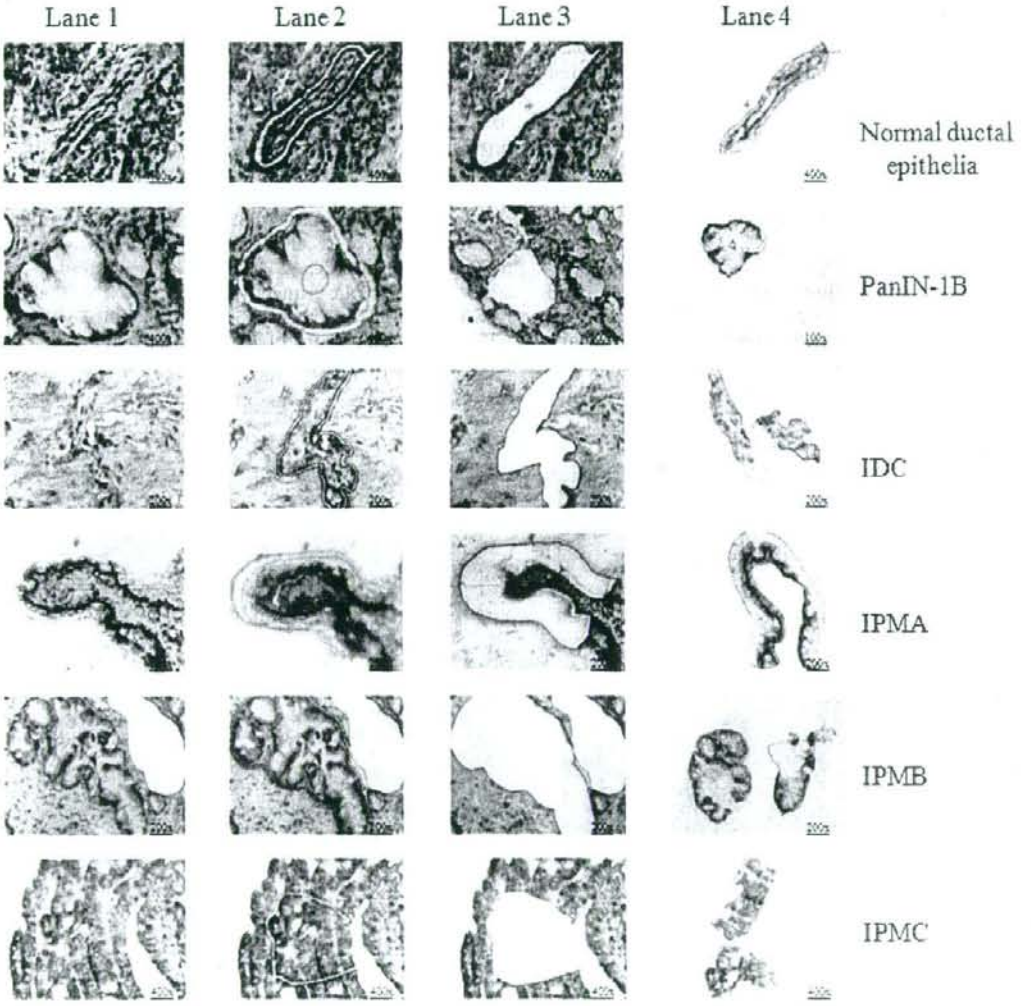

Figure 2
Pictures of pancreatic normal ductal epithelia, PanIN-1B, IDC, IPMA, IPMB and IPMC lesions stained with 1% toluidine blue. Lanes 1 to 4 show the process of microdissecting the target cells. Before cutting (lane 1), after cutting (lane 2), rest of region (lane 3) and target cells in capture (lane 4). (The magnifications are shown on the pictures).

total RNA was extracted from the selected cells and subjected to qRT-PCR for quantitative measurement of *LMO4* as described previously [26].

Statistical analysis

Data were analyzed with multiple comparison in ANOVA (analysis of variance) and Bivariate Correlations with Statistics Package for Social Science (SPSS) software (SPSS Inc. Chicago, IL, USA) after Kolmogorov-Smirnov test to assure that each data set is showed a normal distribution. For multiple comparisons by ANOVA, we used the least significant difference (LSD) test and set the statistical significance at $p < 0.05$. We used Spearman test for bivariate correlations.

Results

Quantitative analysis of LMO4 *expression in 14 pancreatic cancer cell lines, a non-neoplastic ductal epithelial cell line and six primary cultures of pancreatic fibroblasts*

We investigated *LMO4* mRNA expression in 14 pancreatic cancer cell lines, HPDE, a normal pancreatic ductal cell line immortalized by transduction with the E6/E7 genes of human papillomavirus 16 [29,30], and 6 primary cultures of pancreatic fibroblasts derived from resected pancreatic tumors. As shown in Figure 3, 9 of the 14 pancreatic cancer cell lines expressed higher levels of *LMO4* than did HPDE. We also found that all 6 primary cultures of pancreatic fibroblasts expressed moderate levels of *LMO4*. Four pancreatic cancer cell lines, Hs766T, AsPC-1, KP-2 and KP-3, expressed higher levels of *LMO4* than did any of the primary cultures of pancreatic fibroblasts. We next examined whether *LMO4* expression is related to the origin of these cancer cell lines, but found no correlation between *LMO4* expression and site of origin, such as primary or metastatic tumors (Figure 3).

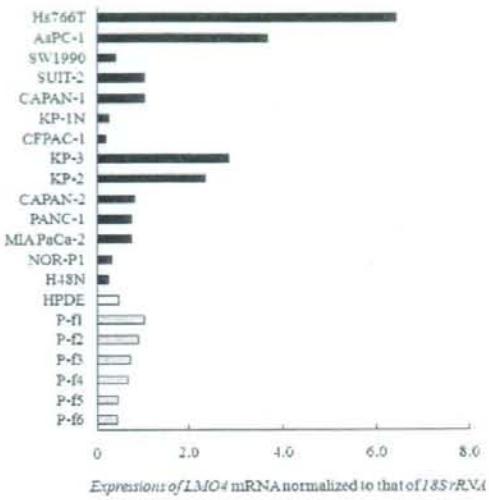

Figure 3
***LMO4* expressions in pancreatic cancer cell lines, a human pancreatic ductal epithelial (HPDE) cell line and primary fibroblasts derived from pancreatic tumors.** Hs766T, AsPC-1, SUIT-2, CAPAN-1, KP-3, KP-2, CAPAN-2, PANC-1 and MIA PaCa-2 cells expressed significantly higher levels of *LMO4* (median, 1.07) than did the HPDE cells (median, 0.46). All 6 cultures of primary fibroblasts express moderate levels of *LMO4* (median, 0.70). The difference in *LMO4* expression between the metastasis group (Hs766T, AsPC-1, SW1990, NOR-P1, SUIT-2 and CAPAN-1; median = 1.06) and the non-metastasis group (KP-2, KP-3, CAPAN-2, PANC-1, MIA PaCa-2 and H48N; median = 0.76) is not statistically significant ($p = 0.75$). Expression of *LMO4* mRNA was normalized to that of *18S* rRNA.

Quantitative analyses of LMO4 *expression in bulk pancreatic tissues*

In the bulk tissue analyses, we measured *LMO4* expression in pancreatic cancer tissues ($n = 11$), and non-neoplastic tissues ($n = 20$), normal pancreatic or chronic pancreatitis-related tissues, and non-malignant IPMN tissues ($n = 11$). As shown in Figure 4, *LMO4* expression was highest in the pancreatic cancer tissues with a mean of 0.24 (95% confidence interval [CI], 0.16 – 0.32), whereas the *LMO4* expression levels were 0.03 (95% CI, 0.02 – 0.04) in non-neoplastic tissues and 0.08 (95% CI, 0.04 – 0.12) in non-malignant IPMN tissues. The mean *LMO4* mRNA level in pancreatic cancer tissues was eight-fold higher than that in non-neoplastic tissues ($p < 0.0001$) and three-fold higher than that in non-malignant IPMN tissues ($p < 0.0001$). The mean *LMO4* mRNA level in non-malignant IPMN tissues was 2.7-fold higher than that in non-neoplastic tissues, although the difference was not statistically significant ($p = 0.053$). All data from bulk tissue analyses indicated that *LMO4* was overexpressed in pancreatic cancer.

Microdissection-based quantitative analysis of LMO4 *expression in IDC, PanIN-2, PanIN-1B and normal ductal cells*

In general, bulk pancreatic tissues are composed of a various types of cells, including ductal epithelial, acinar, islet and mesenchymal cells, and fibroblasts. Cancer cells comprise only 30% – 70% of the cells in bulk tissue specimens of pancreatic cancer [27]. Premalignant cells, such as PanINs, and normal ductal cells comprise even smaller percentages of the cells in non-malignant tissues. The results of our present analyses of cultured cells suggested that *LMO4* was expressed in pancreatic fibroblasts (Figure 3). Therefore, to avoid the influence of contaminating non-ductal cells, we used a laser-microdissection (LMD) method to select specific ductal cells for further analysis.

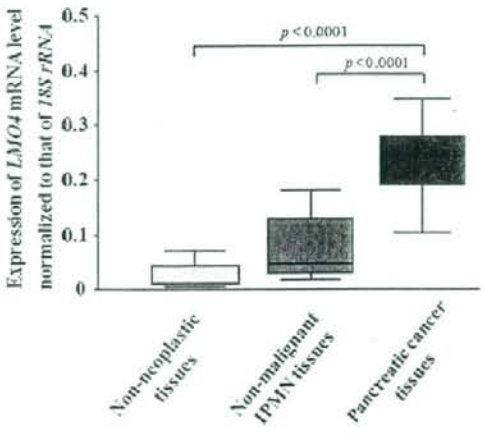

Figure 4
***LMO4* expressions in bulk pancreatic tissues, including 20 non-neoplastic tissues, 11 non-malignant IPMN tissues and 11 pancreatic cancer tissues.** The mean *LMO4* expression value for pancreatic cancer samples is 0.24 (95% CI; 0.16 – 0.32), which is higher than the *LMO4* level of non-malignant IPMN (mean, 0.08; 95% CI, 0.04 – 0.12) or non-neoplastic tissues (mean, 0.03; 95% CI, 0.02 – 0.04). The difference in *LMO4* expression between non-malignant IPMN and non-neoplastic tissues is not statistically significant ($p = 0.053$). The bottom and the top edges of the box mark the lower bound and upper bound of the 95% Confidence Interval for the Mean, respectively. The center horizontal line is drawn at the sample mean. The center vertical lines drawn from the boxes extend to the minimum and the maximum. Expression of *LMO4* mRNA was normalized to that of *18S* rRNA.

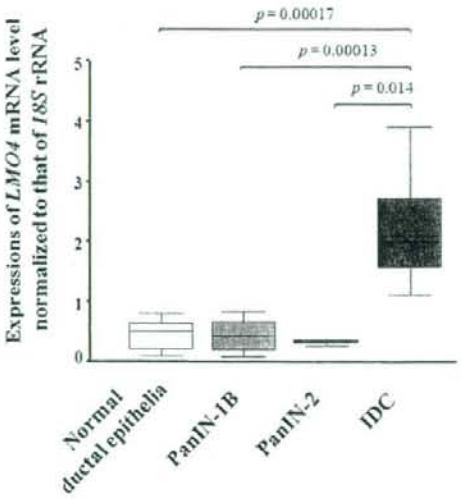

Figure 5
***LMO4* expressions in the PanIN-IDC pathway, including 13 normal ductal epithelial ducts, 14 PanIN-1B lesions, 3 PanIN-2 lesions, and 8 IDC lesions.** IDC cells (mean, 2.19; 95% CI, 1.43 – 2.94) expressed significantly higher levels of *LMO4* than did normal ductal epithelia (mean, 0.45; 95% CI, 0.30 – 0.60), PanIN-1B (mean, 0.41; 95% CI, 0.27 – 0.54), or PanIN-2 cells (mean, 0.32; 95%CI 0.18 – 0.45). The differences in *LMO4* expression among PanIN-2, PanIN-1B, and normal ductal epithelial cells are not statistically significant ($p = 0.54$ and $p = 0.56$). The bottom and the top edges of the box mark the lower bound and upper bound of the 95% Confidence Interval for the Mean, respectively. The center horizontal line is drawn at the sample mean. The center vertical lines drawn from the boxes extend to the minimum and the maximum. Expression of *LMO4* mRNA was normalized to that of *18S* rRNA.

To investigate the involvement of *LMO4* in the PanIN-IDC pathway, we isolated IDC cells from 8 lesions, PanIN-2 cells from 3 lesions, PanIN-1B cells from 14 lesions and normal ductal epithelial cells from 13 ducts by LMD (Figure 2) for quantitative analysis of *LMO4* by RT-PCR. As shown in Figure 5, IDC cells expressed significantly higher levels of *LMO4* than did normal ductal epithelial or PanIN-1B cells ($p < 0.0001$ for both) or PanIN-2 cells ($p = 0.014$). The mean *LMO4* expression level was 2.19 (95% CI, 1.43 – 2.94) in IDC cells, 0.32 (95% CI, 0.18 – 0.45) in PanIN-2 cells, 0.41 (95% CI, 0.27 – 0.54) in PanIN-1B cells and 0.45 (95% CI, 0.30 – 0.60) in normal ductal epithelial cells. The difference in *LMO4* expression between PanIN-2 and normal ductal cells or between PanIN-1B and normal ductal cells was not significant ($p = 0.54$ and $p = 0.56$, respectively). These data suggested that *LMO4* is overexpressed in pancreatic cancer, especially in the invasive step of cancer, but not in the early stage of pancreatic carcinogenesis.

Quantitative analyses of LMO4 *expressions in IPMC, IPMB, IPMA and normal ductal cells*

To investigate the correlation of *LMO4* expression with the IPMA-IPMC pathway, we microdissected IPMC cells from 5 invasive lesions and 3 non-invasive lesions (CIS), IPMB cells from 15 lesions, IPMA cells from 13 lesions and normal ductal epithelial cells from 6 ducts. We then measured *LMO4* expression in these cells by qRT-PCR (Figure 2). As shown in Figure 6, IPMC cells expressed higher levels of *LMO4* (mean, 1.79; 95% CI, 0.99 – 2.58), than did normal ductal cells ($p < 0.001$), IPMA cells ($p < 0.001$) and IPMB cells ($p = 0.003$). There was no signifi-

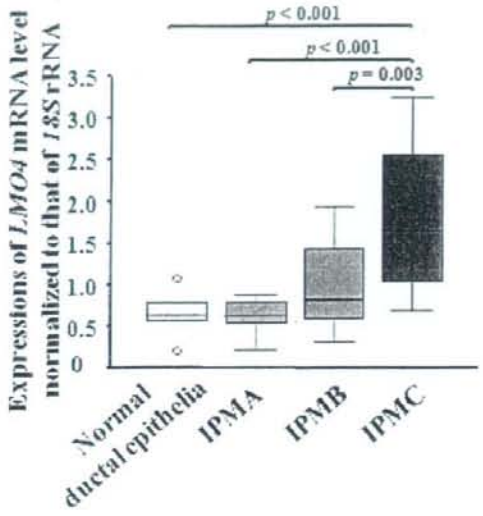

Figure 6
***LMO4* expressions in the IPMA-IPMC pathway, including 6 normal ductal epithelial ducts, 13 IPMA lesions, 15 IPMB lesions and 8 IPMC lesions (5 for invasive IPMC and 5 for non-invasive IPMCs).** IPMC cells expressed significantly higher levels of *LMO4* (mean, 1.79; 95% CI, 0.99 – 2.58) than did IPMB (mean, 0.98; 95% CI, 0.69 – 1.27) ($p = 0.003$), IPMA (mean, 0.60; 95% CI, 0.47 – 0.73) ($p < 0.001$), or normal ductal epithelial cells (mean, 0.65; 95% CI, 0.34 – 0.95) ($p < 0.001$). The differences in *LMO4* expression between IPMB and IPMA cells ($p = 0.09$), IPMB and normal ductal epithelial cells ($p = 0.2$) and IPMA and normal ductal epithelial cells ($p = 0.9$) are not significant. The bottom and the top edges of the box mark the lower bound and upper bound of the 95% Confidence Interval for the Mean, respectively. The center horizontal line is drawn at the sample mean. The center vertical lines drawn from the boxes extend to the minimum and the maximum. Expression of *LMO4* mRNA was normalized to that of *18S* rRNA.

cant difference in *LMO4* expression between invasive IPMC and non-invasive IPMC (CIS). Although the mean *LMO4* expression in IPMB cells (0.98; 95% CI, 0.69 – 1.27) was higher than that in IPMA (0.60; 95% CI, 0.47 – 0.73) or normal ductal cells (0.65; 95% CI, 0.34 – 0.95), the differences in *LMO4* expression were not significant. Taken together, these data suggested that *LMO4* expression may be up-regulated during the late stages of carcinogenesis of IPMN.

Quantitative analyses of* LKB1 *expressions in cultured and microdissected cells, and correlation analyses between* LKB1 *and* LMO4 *in pancreatic carcinogenesis

LKB1 (also called STK11) is a tumor suppressor gene in Peutz-Jeghers syndrome [31]. Loss of this gene is found in pancreatic cancer [32,33]. Recently, LKB1 was reported to induce p21 expression in collaboration with LMO4 [34]. To investigate the potential role of *LMO4* in pancreatic carcinogenesis, we measured *LKB1* mRNA expression in cultured and microdissected cells and investigated any correlation between *LKB1* and *LMO4* expression. In the analysis of cultured cells, we found significant correlation between *LKB1* and *LMO4* mRNA levels in primary cultured fibroblasts (n = 6, spearman test, $p = 0.024$; Figure 7a, top). By contrast, there was no significant correlation between *LKB1* and *LMO4* mRNA levels in cultured cancer cell lines (n = 14, spearman test, $p = 0.33$; Figure 7a bottom). In the analysis of microdissected cells, IDC cells expressed significantly lower levels of *LKB1* mRNA than did PanIN-1B or normal ductal cells (n = 12, $p = 0.02$; n = 8; $p = 0.002$, respectively; Figure 7b top). There was a significant correlation between *LKB1* and *LMO4* mRNA levels in non-malignant cells, including normal pancreatic ductal epithelia and PanIN-1B cells (n = 20, $p = 0.042$, Figure 7b middle), but no significant correlation in IDC cells (n = 8, $p = 0.45$, Figure 7b bottom).

Discussion

In the present study, we performed quantitative real-time RT-PCR to measure *LMO4* expression during pancreatic carcinogenesis through the PanIN-IDC and IPMA-IPMC pathways. Analyses of cultured cells revealed that 9 of 14 pancreatic cancer cell lines and all primary cultures of pancreatic fibroblasts expressed higher levels of *LMO4* than did a non-neoplastic pancreatic ductal cell line. Bulk tissue analysis showed that pancreatic cancer tissues expressed higher levels of *LMO4* than non-neoplastic and non-malignant IPMN tissues; however, the difference in *LMO4* expression between non-neoplastic tissues and non-malignant IPMN was not significant. To avoid the influence of *LMO4*-expressing non-ductal cells contained in bulk tissues, we microdissected target cells, such as IDCs, PanINs, IPMNs and non-neoplastic ductal cells, and measured *LMO4* expression in the microdissected cells. It is usually difficult to obtain frozen sections of intermediate or high-grade PanIN-2 or PanIN-3 (CIS) or non-invasive IPMC (CIS) lesions. In the present study, we obtained frozen sections from 3 cases of PanIN-2 lesions and 3 of non-invasive IPMC. We found that the *LMO4* expression in IDC cells was significantly higher than those in PanIN-1B, PanIN-2, and normal ductal cells; however, the PanIN-2 sample number was small. We also found that both invasive and non-invasive IPMC cells expressed

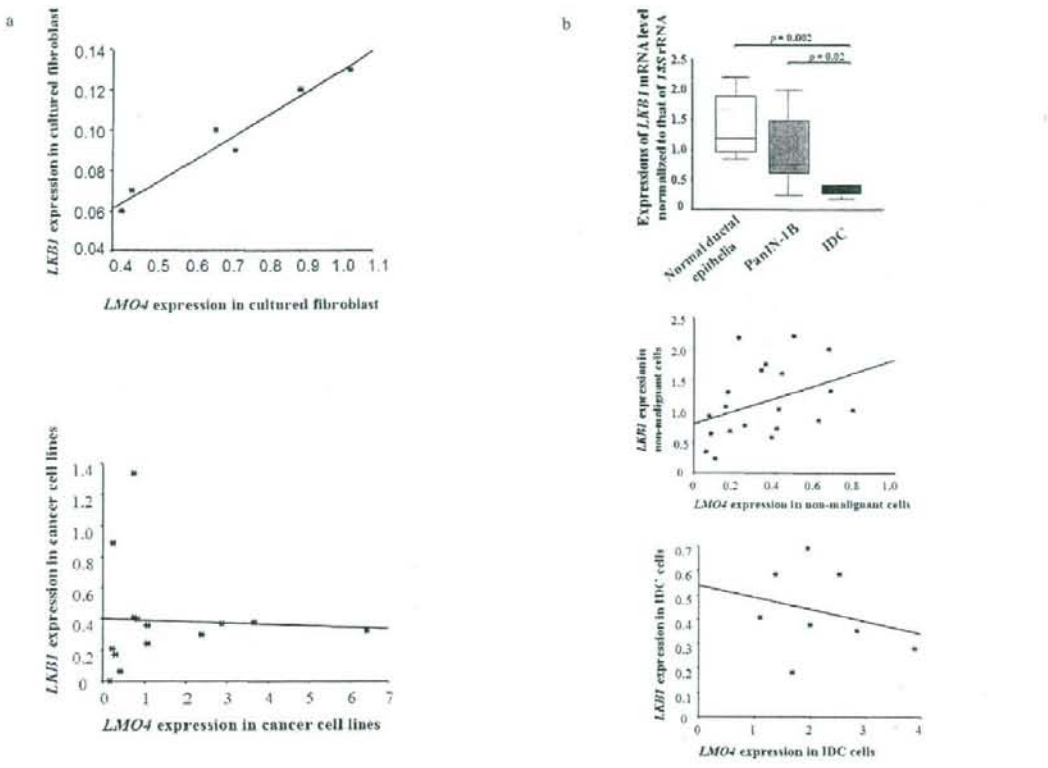

Figure 7

Correlation analyses between *LKB1* and *LMO4* in pancreatic carcinogenesis. a, Correlation between *LKB1* and *LMO4* in primary cultured fibroblasts (spearman test, $p = 0.037$, top) and correlation between *LKB1* and *LMO4* in cultured cancer cell lines (spearman test, $p = 0.39$, bottom). b, Expression of *LKB1* mRNA levels in microdissected normal ducts, PanIN-1B cells, and IDC cells (top). The bottom and the top edges of the box mark the lower bound and upper bound of the 95% Confidence Interval for the Mean, respectively. The center horizontal line is drawn at the sample mean. The center vertical lines drawn from the boxes extend to the minimum and the maximum. Correlation between *LKB1* and *LMO4* mRNA levels in non-malignant cells (including normal ducts and PanIN-1B cells, spearman test, $p = 0.042$, middle), and correlation between *LKB1* and *LMO4* mRNA levels in IDC cells (spearman test, $p = 0.453$, bottom). Expression of *LMO4* mRNA was normalized to that of *18S* rRNA.

higher levels of *LMO4* than did non-malignant IPMN or normal ductal cells. By contrast, we could not detect any differences in *LMO4* expression among PanIN-1B, PanIN-2 and normal ductal cells, or among IPMA, IPMB, and normal ductal cells. Taken together, these data suggested that *LMO4* expression is up-regulated in pancreatic cancer but not in low-grade intraductal precursors in both the PanIN-IDC and IPMA-IPMC pathways.

This is the first report to use qRT-PCR for analyses of *LMO4* expression during pancreatic carcinogenesis. *LMO4* is reported to have an oncogenic role in carcinogenesis and in carcinoma progression in breast cancer and SCC [18-21]. However, the human *LMO4* gene is located on chromosome 1p22.3 [35], which is a region deleted in several human cancers, such as those of liver, skin, and lung [36,37], and Setogawa *et al* reported that the tumor suppressor LKB1 induces p21 expression in collaboration with LMO4, suggesting that LMO4 may have a tumor suppressor function [34]. In the present study, we found that there was significant correlation between *LMO4* and *LKB1* in both primary cultured fibroblasts and microdissected

non-malignant cells, but there was not a significant correlation between *LMO4* and *LKB1* in cancer cells. We also found the downregulation of *LKB1* mRNA in IDC cells, consistent with LKB1's tumor suppressive function. Our data suggest that alteration of the LKB1-LMO4 balance is involved in pancreatic carcinogenesis, although the exact function of LMO4 in pancreatic carcinogenesis remains unknown. Taken together, there appears to be a conflicting function of LMO4 in carcinogenesis as a tumor suppressor or as an oncogene; it is reasonable that LMO4, a transcription regulator, may have multiple functions in individual cancers, like E2F1, an another transcription factor, that was reported both as an oncogene by stimulating cell proliferation [38] and as a tumor suppressor by signaling p53-dependent apoptosis [39].

Recently, Murphy *et al* [40] used immunohistochemical staining and reported that a subset of patients with low LMO4 expression-pancreatic cancers had poor outcomes. In the present study, *LMO4* mRNA was not overexpressed in any of the 14 pancreatic cancer cell lines. However, all IDC cells microdissected from cancer tissues showed relatively high expression of *LMO4* mRNA although the sample number was small. This might have been the result of using of frozen sections with a histological diagnosis of moderately or well-differentiated adenocarcinoma, which can be microdissected easily. Patients with well-differentiated adenocarcinoma usually have better prognosis [41]; thus the present data may be partially consistent with Murphy's result demonstrating that high LMO4-pancreatic cancers are associated with a significant survival advantage for patients with surgical resection.

Taken together, it remains unclear if LMO4 has an oncogenic function or a tumor suppressive function in pancreatic carcinogenesis. To better clarify the functional roles of LMO4 in pancreatic carcinogenesis, further examinations such as inhibition experiments using RNAi technology are needed.

In conclusion, the present results showed that *LMO4* is overexpressed in pancreatic cancer related to both the PanIN-conventional IDC pathway and the IPMA-IPMC pathway, but not at the early stages of pancreatic carcinogenesis.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JY participated in the design of the study, carried out the RNA expression analysis, Laser Capture Microdissection studies, statistical analysis, and drafted the manuscript. KO and KM participated in the design and coordination of the study, performed the statistical analysis, and critically revised the manuscript. LC performed realtime expression analysis and critically revised the manuscript. KN and HY participated in histologic examination and critically revise the manuscript. HF, TE and HK performed the statistical analysis and critically revised the manuscript. MT planned and coordinated the study, and critically revised the manuscript. All authors have read and approved the final version of the manuscript.

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C/EBP homologous protein is crucial for the acceleration of experimental pancreatitis

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Abstract

C/EBP homologous protein (CHOP) is one of the main mediating factors in the ER stress pathway. To elucidate the role of the ER stress-CHOP pathway in experimental pancreatitis, wild-type ($Chop^{+/+}$) and *Chop* deficient ($Chop^{-/-}$) mice were administered cerulein, a cholecystokinin analogue, or both cerulein and lipopolysaccharide (LPS). In cerulein-induced acute pancreatitis, ER stress, serum amylase elevation and histological interstitial edema were induced. However, there was no remarkable activation downstream of the CHOP pathway regardless of the presence or absence of CHOP. Whereas, in the cerulein and LPS model, inflammation-associated caspases (caspase-11, caspase-1) and IL-1β, but not apoptosis-associated caspases, were activated. In $Chop^{-/-}$ mice, the expression levels of these mediators returned to basal levels resulting in a milder pancreatitis and decreased serum amylase level. These results indicated that the ER stress-CHOP pathway has a pivotal role in the acceleration of pancreatitis through the induction of inflammation-associated caspases and IL-1β.

Keywords: Acute pancreatitis; ER stress; BiP; XBP-1; CHOP; IL-1β; LPS; Apoptosis; Caspase

One of the most important pathological states of the exocrine pancreas is acute pancreatitis, which initially arises in the pancreatic acinar cells by mechanisms that are incompletely understood [1]. After initiation, the disease spreads systemically through the development of an inflammatory response driven by proinflammatory cytokines [2]. Along with proinflammatory mechanisms, protective and restorative mechanisms are also activated in the stressed acinar cells. Thus, the ultimate severity of acute pancreatitis depends on the balance of these opposing forces.

Pancreatic acinar cells have the highest rate of protein synthesis among all human tissues [3]. Because of this prominent role in digestive enzyme synthesis, pancreatic acinar cells contain particularly abundant ER. Thus, pancreatic acinar cells appear susceptible to perturbations of ER homeostasis. Indeed, secretagogue treatment of isolated rat pancreatic acini leads to the activation of the ER stress response in pancreatic acinar cells [4]. As well, all major ER stress sensing and signaling mechanisms have been shown to be activated in the exocrine acini of the arginine model of acute pancreatitis [5].

ER performs several important functions, including posttranslational modification such as folding, and the assembly of newly synthesized secretory or cell membrane proteins. Its proper function is essential to ensure cell survival [6,7]. ER has an especially important function in the survival of hepatocyte and pancreatic acinar cells, as well as in the differentiation of plasma cells [8]. When the cells

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are subjected to ER stress, several pathways are activated to protect the cells [9,10]. These responses involve induction of ER stress-associated factors, including Ig H chain binding protein (BiP), X-bix binding protein (XBP1) and C/EBP homologous protein (CHOP). BiP and XBP1 are good markers of the activation of the ER stress pathway, function as intracellular stress sensors, and protect cells from various stresses. However, when the ER functions are severely impaired, CHOP is induced by ER stress, leading to apoptosis [11].

Recently, the significance of ER stress in various inflammatory diseases has come under discussion. Endo et al. reported that CHOP plays a crucial role in the pathogenesis of inflammation through the induction of caspase-11 [12,13]. They showed that LPS-induced inflammation in the lung, including IL-1β activity in bronchoalveolar lavage fluid, was attenuated in *Chop*$^{-/-}$ mice. Various cytokines, produced from macrophages and other cells, are involved in the pathogenesis of many inflammatory diseases. IL-1β is secreted at an early stage of the inflammatory response and plays a triggering role in inflammatory reactions [13]. In macrophages with activated CHOP as a result of ER stress, procaspase-11 is processed by autoproteolysis. The active form of caspase-11 proteolytically converts procaspase-1 to caspase-1, which then activates pro-IL-1β to IL-1β [14]. Because both caspase-11 and canspase-1 play a pivotal role in the initiation of inflammatory reactions, they are called inflammation-associated caspases [13]. IL-1β is secreted after activation from pro-IL-1β, and has an important function in the initial process of the inflammatory reaction. On the other hand, apoptosis-associated caspases, caspase-3 and caspase-9, were not apparently activated. These observations suggest that CHOP may have a critical role in the pathogenesis of inflammatory reactions.

In this study, we examined whether the ER stress-CHOP pathway is involved in the pathogenesis and acceleration of acute experimental pancreatitis, and whether inflammation-associated caspases and apoptosis-associated caspases are activated in acute pancreatitis.

Materials and methods

Generation and genotyping of animal models. All procedures were approved by the Animal Care and Use Committee of Kumamoto University. Mice lacking the *Chop* gene (C57BL/6J-Chop^{tm1}; *Chop*$^{-/-}$) were generated as previously described [15]. The genotype for *Chop* was determined by PCR. For detecting the wild allele, the following primers were used: 5′-CCTGGATTAAGCTTGGTAGT-3′ as the sense primer, and 5′-GGACGCAGGGTCAAGAGTAG-3′ (derived from the *Chop* gene) as the antisense primer. For detecting the knockout allele, the following primers were used: 5′-GAGAAAAAAAGAGTACAAATG GCCTGG-3′ (derived from the *Chop* gene), as the sense primer and 5′-A TCGCCTTCTATCGCCTTCTTGACGAG-3′ (derived from the neomycin resistant gene) as the antisense primer. Thermal cycle reaction was performed as follows: 94 °C for 5 min, followed by 30 cycles at 94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s, and 72 °C for 7 min. The wild-type and knockout alleles yielded 1.1 kb and 0.8 kb transcripts, respectively.

Cerulein-induced pancreatitis/cerulein-induced and LPS-accelerated pancreatitis (cerulein + LPS pancreatitis). In this experiment, wild-type C57BL/6J mice (*Chop*$^{+/+}$), *Chop*$^{-/-}$ mice were used. After overnight fasting, female mice (7–8 weeks old and weighing 19–23 g) were given hourly intraperitoneal injections of saline as control ($n = 3$) or a supramaximal stimulating concentration of cerulein dissolved in saline (50 μg/kg; $n = 3$) (Sigma–Aldrich Corp, Tokyo, Japan) for several hours (6–12 h). One hour after the last injection, mice were killed and the serum and pancreas rapidly prepared for study. The serum was used for measurement of amylase activity. The pancreas was used for Western blot and RT-PCR analysis. Lipopolysaccharides (LPS, *Escherichia coli* 0111, Sigma–Aldrich Corp.) was injected three times; at the beginning of overnight fasting, at the same time as the first and twelfth cerulein injections, respectively.

Histological analysis. For histological analysis, pancreatic tissue was fixed overnight in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin & eosin (H&E).

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling assay. For the detection of apoptosis, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay was performed using an *in situ* apoptosis detection kit (Wako, Osaka, Japan).

RT-PCR analysis. Mice were killed at the indicated times after treatment. Pancreases were removed and stored at −80 °C. Total RNA from pancreas was isolated using the acid guanidium thiocyanate phenol–chloroform extraction procedure as described [16]. cDNA was synthesized using a Superscript one-step RT-PCR system (Invitrogen Life Technologies, Carlsbad, CA). The primers used for PCR were as follows: sense primer, 5′-ACTCATTGTGGCTGTGGAGAAG-3′ and antisense primer, 5′-GCCGTCTTTCATTACACAGGAC-3′ for IL-1β (GenBank Accession No. M15131); sense primer, 5′-ACACGTCTTGCCCTCATTAT CTGC-3′ and antisense primer, 5′-CCACTCCTTGTTTCTCTCCACG-3′ for caspase-1 (GenBank Accession No. NM009807); sense primer, 5′-GC GTTGGGTTTTTGTAGATGCC-3′ and antisense primer, 5′-ATGTGC TGTCTGATGTCTGGTG-3′ for caspase-11 (GenBank Accession No. Y13089); sense primer, 5′-GAAAGGATGGTTAATGATGCTGAG-3′ and antisense primer, 5′-GTCTTCAATGTCCGCATCCTG-3′ for BiP (GenBank Accession No. AJ002387); sense primer, 5′-CATACACCACC ACACCTGAAAG-3′ and antisense primer, 5′-CCGTTTCCTAGTT CTTCCTTGC-3′ for CHOP (GenBank Accession Number X67083). The primer sets for IL-1β, caspase-1, caspase-11, BiP, and CHOP were expected to give PCR products of 384, 372, 439, 231, and, 357 bp, respectively. PCR consisted of an initial denaturation cycle at 94 °C for 5 min, followed by the 30 cycles at 94 °C for 15 s, annealing at 55 °C for 30 s, and elongation at 68 °C for 1 min. An additional cycle at 72 °C for 7 min completed the amplification process. The primers used for detection of G3PDH mRNA were as follows: sense primer, 5′-GGAAAGC TGTGGCGTGATG-3′ and antisense primer, 5′- CTGTTGCTGTA GCCGTATTC-3′. The primers used for detection of XBP-1 mRNA were as follows: sense primer, 5′-AAACAGAGTAGCAGCGCAGACTGC-3′ and antisense primer, 5′-GGATCTCTAAAACTAGAGGCTTGGTG-3′. The primer sets for G3PDH and XBP-1 were expected to give PCR products of 380 and 600 bp, respectively. PCR consisted of an initial denaturation cycle at 94 °C for 5 min, followed by 27 cycles consisting of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, and elongation at 72 °C for 30 s. An additional cycle at 72 °C for 7 min completed the amplification process. Amplified PCR products were separated by 1% agarose gel electrophoresis and visualized by ethidium bromide staining.

Serum amylase activity. Substrate for measurement of pancreatic amylase activity was 2-chloro-4-nitrophenyl-4-galactopyranosylmaltoside (Gal-G2-CNP) (CicaLiquid-N p-AMY, Kanto Chemical Co., Inc. Tokyo, Japan).

Western blot analysis. Pancreas samples were homogenized in lysate buffer (Hepes 50 mmol/L, pH 7.4, NaCl 150 mmol/L, Triton X-100 0.1%, glycerol 10%, NaF 1 mmol/L, sodium orthovanadate 2 mmol/L, ethylenediaminetetraacetic acid 1 mmol/L, and a protease inhibitor cocktail [1:100 dilution; Sigma–Aldrich]). Extracts (12–15 μg of protein per lane) were applied to 12% polyacrylamide gel electrophoresis and transferred to an Immobilon polyvinylidene difluoride filter (Millipore, Billerica, MA). Primary antibodies to the following antigens (made in rabbit) were used at the indicated dilutions: Caspase-3 (Cell Signaling Technology, Inc. Danvers, MA), 1:1000; Caspase-9 (Cell Signaling Technology), 1:1000; and

Actin (Sigma–Aldrich Corp.) 1:1000. An anti-rabbit immunoglobulin G antibody conjugated with horseradish peroxidase (Amersham Biosciences Corp., Piscataway, NJ) was used for detection. Unpaired Student's t tests were used to calculate P values.

Results

Pancreatitis in Chop$^{+/+}$ and Chop$^{-/-}$ Mice

To analyze the relationship between the severity of pancreatitis and the ER-CHOP pathway, we induced experimental pancreatitis by cerulein or by cerulein + LPS. In saline-treated mice, no pathologic changes were observed in the histology of pancreas of *Chop*$^{+/+}$ and *Chop*$^{-/-}$ mice (Fig. 1A and B). Both *Chop*$^{+/+}$ and *Chop*$^{-/-}$ mice treated with cerulein showed evidence of interstitial edema and the accumulation of a huge number of vacuole-like structures distributed throughout the cytoplasm (Fig. 1C and D). The degree of pancreatic inflammation in *Chop*$^{-/-}$ mice was almost the same as in *Chop*$^{+/+}$ mice. Using both cerulein and LPS, Ding et al. reported the successful establishment of a mouse model for severe pancreatitis [17]. We used this method to investigate whether *Chop* is involved in this model. Severe inflammatory changes, such as the destruction of acinar cells and the infiltration of inflammatory cells were diffusely observed in the pancreas of *Chop*$^{+/+}$ mice treated with cerulein + LPS (Fig. 1E). However, such inflammatory changes were obviously attenuated in the pancreas of *Chop*$^{-/-}$ mice (Fig. 1F).

Amylase activity in Chop$^{+/+}$ and Chop$^{-/-}$ mice

To assess the degree of pancreatitis, we analyzed the serum amylase activity of *Chop*$^{+/+}$ and *Chop*$^{-/-}$ mice in which had been induced pancreatitis by cerulein or cerulein + LPS. In *Chop*$^{+/+}$ mice, serum amylase levels were 8085 ± 1761 IU/L ($n = 2$) and $10{,}690 \pm 692$ U/L ($n = 3$) after treatment with cerulein or cerulein + LPS, respectively, while the serum level from saline treatment was 940 ± 35 U/L ($n = 3$) (Fig. 2A). There was a tendency for the induction levels of serum amylase from cerulean + LPS to be higher than that from cerulein. In *Chop*$^{-/-}$ mice, the serum amylase levels were 7940 ± 482 IU/L ($n = 3$) and 7920 ± 1032 U/L ($n = 3$) from treatment with cerulein or cerulein + LPS, respectively; while the serum level from saline treatment was 990 ± 159 U/L ($n = 3$) (Fig. 2B). Thus, there was no significant difference between the induction levels of serum amylase in *Chop*$^{+/+}$ and *Chop*$^{-/-}$ mice after stimulation with cerulein. But the serum amylase levels returned to the same level as those found in *Chop*$^{+/+}$ mice with cerulein treatment in the absence of CHOP. Thus, there was a significant difference ($p < 0.01$) between serum amylase levels in *Chop*$^{-/-}$ and *Chop*$^{+/+}$ mice when treated with cerulein + LPS. These results indicated that

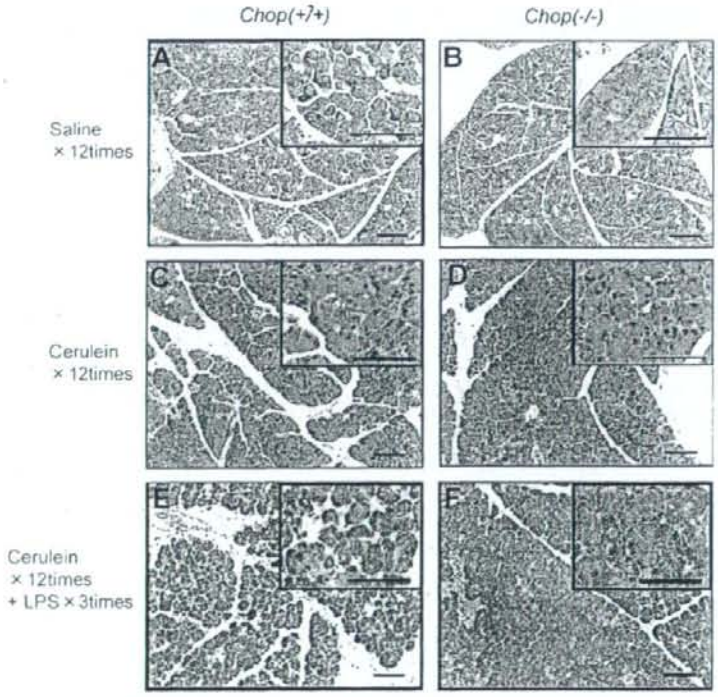

Fig. 1. Histological analysis of experimental pancreatitis in *Chop*$^{+/+}$ and *Chop*$^{-/-}$ mice. Scale bars: 100 μm. (A, B) Saline-treated group. (C, D) Cerulein-treated group. (D, E) Cerulein + LPS treated group.

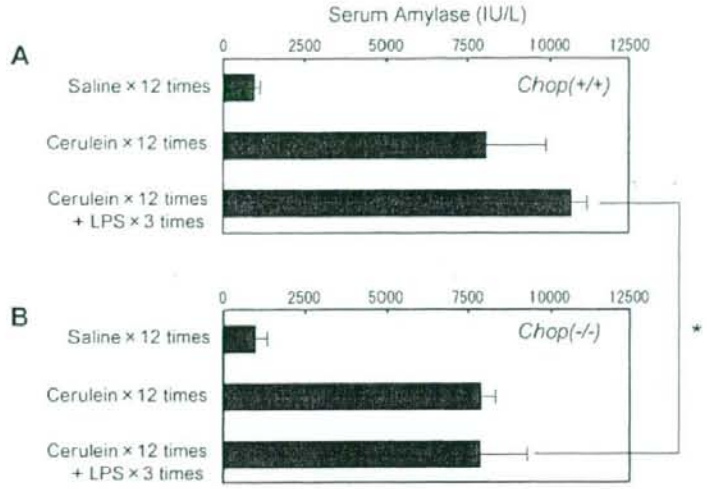

Fig. 2. Analysis of serum amylase activities in $Chop^{+/+}$ and $Chop^{-/-}$ mice of each experimental model of pancreatitis. (A) $Chop^{+/+}$ mice (B) $Chop^{-/-}$ mice In $Chop^{-/-}$ mice treated by cerulein + LPS, the induction of serum amylase is suppressed significantly compared with $Chop^{+/+}$ mice treated by cerulein + LPS ($^*P < 0.01$; unpaired Student's t test).

the CHOP pathway is involved in the severe pancreatitis induced by cerulein + LPS.

Induction of ER stress, inflammatory mediators and apoptotic caspases in $Chop^{+/+}$ and $Chop^{-/-}$ mice

ER stress related mechanisms were evaluated after treatment by cerulein or by cerulein + LPS. BiP, XBP-1 and CHOP were activated in $Chop^{+/+}$ mice when treated by cerulein or cerulein + LPS (Fig. 3A). Levels of BiP and XBP-1 were increased also in $Chop^{-/-}$ mice. These results indicated that ER stress is induced by cerulein or cerulein + LPS, regardless of the presence or absence of CHOP.

In $Chop^{+/+}$ mice, caspase-11, caspase-1 and IL-1β were not activated in the cerulein-treated group. However, these inflammatory mediators were increased in $Chop^{+/+}$ mice when treated with cerulein + LPS (Fig. 3A). On the other hand, the expression levels of these inflammatory mediators were much lower in $Chop^{-/-}$ mice. These results indicated that the induction of caspase-11, caspase-1 and IL-1β was mediated by CHOP, and that the milder pancreatitis induced by cerulein + LPS in $Chop^{-/-}$ mice was due to the suppression of IL-1β.

CHOP expression is induced at high levels by ER stress, and CHOP induces apoptosis through activation of caspase-9 and caspase-3 [11]. To investigate whether the CHOP-apoptotic pathway is related to the attenuation of pancreatitis in $Chop^{-/-}$ mice, the expression levels of caspase-9 and caspase-3 were analyzed in each model of experimental pancreatitis. In the cerulein-treated group, there was no induction of caspase-9 and caspase-3 in $Chop^{+/+}$ and $Chop^{-/-}$ mice. In the cerulein + LPS treated group, there was a slight induction of caspase-9 and caspase-3 in $Chop^{+/+}$ and $Chop^{-/-}$ mice (Fig. 3B).

The results of TUNEL assay are shown in Fig. 4. TUNEL positive cells were not observed in the saline-treated $Chop^{-/-}$ and $Chop^{+/+}$ mice. In $Chop^{+/+}$ mice, the number of TUNEL positive cells in the cerulein + LPS treated group was higher than that in the cerulein-treated group. In $Chop^{-/-}$ mice treated with cerulein + LPS, the number of TUNEL positive cells decreased to the same level as that in $Chop^{+/+}$ mice (Fig. 4B). These results suggested that apoptosis was actually induced, although there was no difference in the expression levels of the apoptosis-associated caspases, caspase-9 and caspase-3.

Discussion

Here we showed that the ER stress-CHOP pathway has an important function in the acceleration of acute pancreatitis through the activation of the inflammation-associated caspases pathway, caspase-11, caspase-1 and IL-1β.

We demonstrated the activation of the ER stress pathway as an early event during the development of acute pancreatitis in the cerulein mouse model. Although BiP and XBP-1 were activated by cerulein, there was no difference in the severity of pancreatitis, levels of amylase, or numbers of TUNEL positive cells between $Chop^{+/+}$ and $Chop^{-/-}$ mice. Indeed, CHOP-mediated inflammatory and apoptosis pathways were not activated, as demonstrated by the unchanged levels of caspases and IL-1β. These results suggested that CHOP-mediated pathways are not involved in cerulein pancreatitis.

To analyze the role of CHOP in more detail, we used the more severe pancreatitis model from cerulein and LPS [17]. LPS is a kind of endotoxin that can activate the mononuclear cell system to release cytokines. These cytokines can activate the production of inducible nitric oxide (NO) syn-

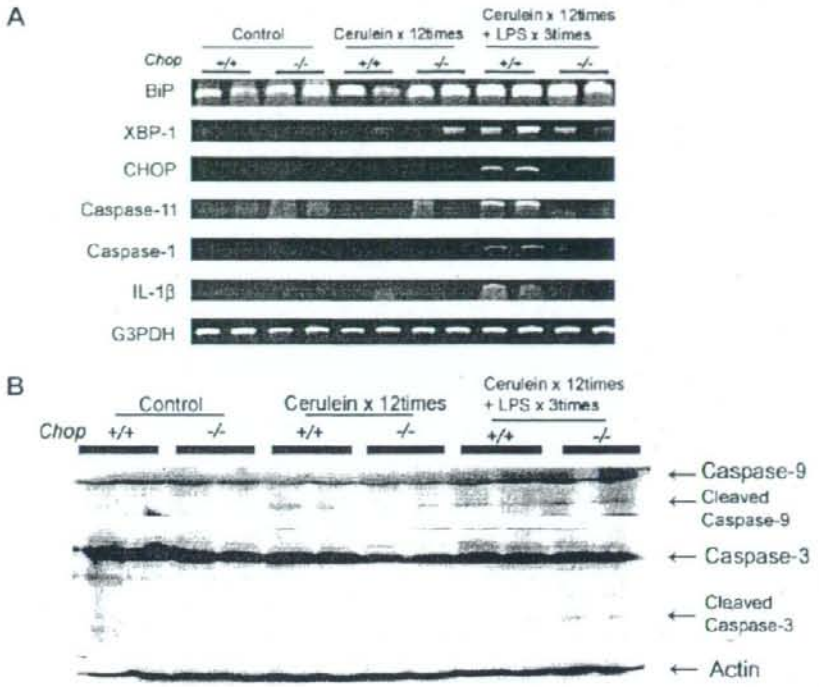

Fig. 3. Analysis of ER stress, inflammatory mediators, and apoptotic caspases in $Chop^{+/+}$ and $Chop^{-/-}$ mice of each experimental model of pancreatitis. A) RT-PCR analyses of ER stress-associated factors and inflammatory mediators. (B) Western blot analyses of caspase-9 and caspase-3.

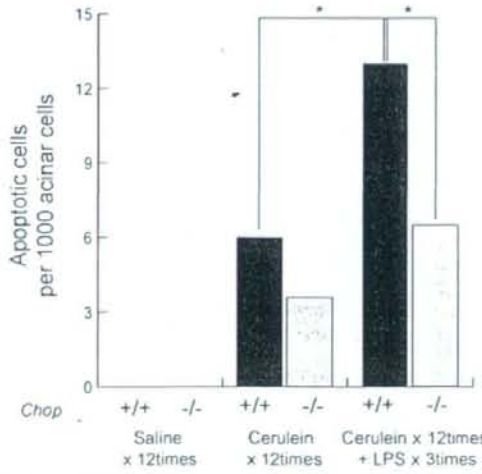

Fig. 4. TUNEL assay in $Chop^{+/+}$ and $Chop^{-/-}$ mice of each experimental model of pancreatitis ($^*P < 0.05$; unpaired Student's t test).

chase, resulting in the overproduction of NO. As NO acts as an endothelium-derived relaxing factor and a highly reactive free radical, excessive production of NO causes vasodilatation and increased microvascular permeability, resulting in cellular damage. Thus, treatment with both cerulein and LPS can cause severe pancreatitis in mice. Like in cerulein pancreatitis, BiP and XBP-1 were activated by treatment with cerulean and LPS. The ER stress-CHOP pathway is believed to induce inflammation-associated caspases (caspase-11 and caspase-1). In fact, as shown in Fig. 3, while these caspases and IL-1β were activated in $Chop^{+/+}$ mice, they were considerably down-regulated in $Chop^{-/-}$ mice. The expression of caspase-11 activates caspase-1 and the complex of activated caspase-1 and caspase-11 increases the activation of pro-IL-1β to mature IL-1β. Activated IL-1β is considered to trigger the inflammatory cytokines cascade [13]. In $Chop^{-/-}$ mice, this inflammation cascade through IL-1β was blocked. These results showed that the severity of pancreatitis was attenuated and the level of amylase decreased to the same level as seen in cerulein model in $Chop^{-/-}$ mice. These results also suggest that the CHOP-mediated inflammatory pathway has a major role in the severe pancreatitis induced by cerulein and LPS.

Although treatment with cerulein and LPS-induced apoptosis-associated caspases (caspase-3 and caspase-9), their levels were very low even in the presence of CHOP. Nevertheless, TUNEL positive cells were increased in $Chop^{+/+}$ mice, and decreased in $Chop^{-/-}$ mice. These results suggested that the enhanced apoptosis induced by cerulein and LPS was mediated by CHOP, but not through apoptosis-associated caspases. A similar situation was reported by Endo et al. [12]. In the mouse model of LPS-induced pneu-

monia, a blockade of apoptosis-associated caspases in *Chop*$^{-/-}$ mice was small, but the pneumonia itself was attenuated compared with that in *Chop*$^{+/+}$ mice [12]. Thus, induction of TUNEL positive apoptotic cells may be a reflection of IL-1β induction, another pro-apoptotic molecule. Although the major function of caspase-11 and caspase-1 is believed to regulate cytokine maturation, the function of these caspases is not restricted to the inflammatory cascade [18,19]. Caspase-11 can mediate the activation of both caspase-1 and caspase-3. While caspase-3 mediates induction of apoptosis, caspase-1 is a regulator of apoptosis. Thus, the regulation of the two downstream caspases by caspase-11 may contribute to the development of apoptosis under pathological conditions [19]. IL-1β also promotes the cell death pathway [20].

Concerning the role of apoptosis in experimental pancreatitis, it is generally believed that acinar cells die through both necrosis and apoptosis in experimental models of acute pancreatitis [21,22]. The apoptosis/necrosis ratio varies in different experimental models of pancreatitis. In mouse, the severity of experimental pancreatitis directly correlates with the extent of necrosis and inversely with that of apoptosis [22–24]. Mareninova et al. [25] analyzed the cell death pathway in cerulein pancreatitis and demonstrated that the high necrosis and little apoptosis in the mouse model of cerulein pancreatitis was due to caspases inactivation by XIAP (X-linked inhibitor of apoptosis protein). When a caspase was induced by XIAP inhibitor, embelin, stimulation of apoptosis and decreased necrosis was observed, resulting in normalization of pancreatic histology. In our case, acute pancreatitis became less severe in the absence of CHOP, although apoptosis also decreased. This is apparently contrary to the data described above; that induction of apoptosis can reduce the severity of pancreatitis in mouse. This can be explained as follows. Our data demonstrated that the processing of apoptosis-associated caspases was not observed even in the presence CHOP in the cerulein and LPS model. Similar data that apoptosis-associated caspases were not activated in a cerulein model was reported by Mareninova et al. [25]. On the other hand, we showed that the inflammatory-associated pathway was activated by cerulein + LPS and that this pathway was indeed substantially inhibited in the absence of CHOP. Taken together, these results suggested that the level of apoptosis is too low to influence the severity of pancreatitis induced by cerulein and LPS. Thus, a decrease in apoptosis does not affect the severity of pancreatitis. However, it is possible that enhancement of apoptosis increases the apoptosis/necrosis ratio, resulting in the reduction of the severity of pancreatitis.

To the best of our knowledge, this is the first report showing that the CHOP-caspase-11 pathway has an important role in the acceleration of pancreatitis *in vivo*. Severe acute pancreatitis can be fatal, and the CHOP-inflammation-associated caspases pathway may thus become a new target for therapy against this disease.

Acknowledgments

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Long-term outcomes of extended radical resection combined with intraoperative radiation therapy for pancreatic cancer

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Abstract

Background/Purpose. Systemic and/or local recurrence often occurs even after curative resection for pancreatic cancer (PC). To prevent local relapse we adopted an extended radical resection combined with intraoperative radiation therapy in patients with PC, and all the patients were followed for more than 5 years.

Methods. We assessed the long-term outcomes of 41 patients who underwent this combined therapy. The cumulative survival curve in this series was depicted using the Kaplan-Meier method. Statistical analyses were performed using the log-rank test.

Results. The actual 5-year survival rate was 14.6%, with a median survival time of 17.6 months. Six patients have been 5-year survivors. Local recurrence occurred in only 2 patients (5.0%). Cancer-related death occurred in 32 patients, 18 of whom had liver metastases. The patients with liver metastases had a significantly shorter survival time than those with other cancer-related causes of death. Patients with n3 lymph node involvement, extrapancreatic nerve plexus invasion, and stage IV disease had significantly poorer prognoses than patients without these characteristics.

Conclusions. Our combined therapy for patients with PC contributed to local control; however, it provided no survival benefit, because of liver metastases.

Key words Pancreatic cancer · Actual 5-year survival · Extended radical resection · Intraoperative radiation therapy · Multimodality treatment

Introduction

Pancreatic cancer (PC) remains a lethal disease, in which the annual incidence is approximately equal to the annual deaths.[1] Surgical resection offers the only chance for cure, but even after curative resection there is a high probability of systemic and/or local relapses. Local recurrence occurs in 71.8%–73% of the patients.[2,3] One reason is that even when small tumors are located in the head or neck of the pancreas, they are frequently in close proximity to the superior mesenteric artery (SMA) or the celiac axis (CA), often yielding surgical margins measured in millimeters or less. A second reason is that the invasion of periarterial nerve sheaths could mean that there is a positive resection margin.[4] Actually, autopsies of patients who had standard resections for PC showed that local recurrence occurred in the soft tissue around the SMA and CA.[5] Therefore local control is one of the most crucial points of treatment for PC.

We applied intraoperative radiation therapy (IORT) in 1969[6] and first utilized IORT combined with resection in 1976[5,7] for PC. The rationale for the use of IORT is to maximize the dose of radiation delivered to the tumor bed and around the aorta (where cancer cells might invade) and to minimize the radiation dose to surrounding normal tissues. Studies regarding the involvement of lymph nodes have confirmed that patients undergoing the standard Whipple procedure have positive nodes outside the dissection.[8]

We adopted extended radical resection combined with IORT for PC in 1984. All patients who underwent this combined therapy have been followed more than 5 years after surgery. Our aim in this study was to assess the long-term outcomes of this combined therapy for PC.

Patients and methods

Patients and surgical technique

Between December 1984 and December 1999 at Kumamoto University, 41 patients underwent extended

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radical pancreatectomy combined with IORT. Written informed consents were obtained from all the patients before the treatment.

This combined therapy involves the dissection of the juxta-paraaortic and regional lymph nodes together with the connective tissue and nervous plexus around the aorta, extending from the diaphragm above to the inferior mesenteric artery below. Following dissection, a dose of 30 Gy with 9- to 12-Mev electron-beam radiation was administered to the operative field, using a special variable pentagon applicator which could change the radiation field depending on the body size and the area of the tumor bed, including the paraaortic area from the diaphragm above to the inferior mesenteric below, as described previously.[5]

Histopathological examination and outcomes analyzed

We evaluated histological characteristics according to classification of PC defined by the Japan Pancreas Society.[9]

Local recurrence and/or distant metastases were defined as the detection of apparent mass formation on imagings. For the evaluation of recurrences, all patients were evaluated every 3 months for the first year after surgery, every 4 months the second year, and every 6 months subsequently, by chest and abdominal computed tomography (CT). Cytology or histological confirmation of recurrent disease was not required. The first site of recurrent disease was documented for the outcome analyses. Hospital death was defined as death during hospitalization.

Statistical analysis

The Kaplan-Meier method was used to analyze survival, and levels of significance were tested with the log-rank test. Differences were considered to be significant at $p < 0.05$.

Results

Patient characteristics

The study subjects included 17 women and 24 men. The median age of the patients was 60 years (range, 37 to 76 years). The primary pancreatic lesion was located in the head in 32 patients, in the body in 8, and in the tail in 1. We performed a pylorus-preserving pancreaticoduodenectomy in 14, a Whipple resection in 17, a distal pancreatectomy in 7, and a total pancreatectomy in 3. Histological examination could be performed in 40 patients. The histological characteristics are listed in Table 1. Of the 40 patients, 27 (67.5%) were affected by stage IV disease. The median tumor size was 3.0 cm (range, 1.1 to 12.0 cm). Lymph node metastases (n) were identified in 33 (82.5%) patients. Portal vein invasion (pv) was found in 14 (35.0%) patients. As to residual tumor (R), an R0 resection was performed in 26 (65.0%) patients, an R1 resection in 10, and an R2 resection in 4. Extrapancreatic nerve plexus invasion (pl) was found in 18 (45.0%) patients.

Table 1. Histological characteristics

No. of patients	40
Stage	
II	1
III	12
IVa	15
IVb	12
Tumor size (cm)	
1.0–2.0	3
2.1–4.0	27
4.1<	10
Nodal involvement	
n0	7
n1	17
n2	12
n3	4
Residual tumor	
R0	26
R1	10
R2	4
Portal vein invasion	
Present	14
Absent	26
Extrapancreatic nerve plexus invasion	
Present	18
Absent	22

Outcomes

Figure 1 shows the cumulative survival curve for patients with the combined therapy. The actual 5-year survival rate was 14.6%, with a median survival time of 17.6 months. Two patients are still alive without recurrence, 95.4 and 254.5 months after surgery, respectively. Six patients survived for more than 5 years. The characteristics of the 5-year survivors are outlined in Table 2. One patient was in stage II, two in stage III, and two in stage IVa. One patient had n0, four had n1+, and one had n2+. Three patients died of cancer relapses more than 5 years after the surgery.

The outcomes of all the patients are summarized in Table 3. Cancer-related death was observed in 32 patients, 18 of whom had liver metastases. Local recurrence was observed in only 2 patients (5.0%), although autopsies disclosed microscopic local recurrence in 4 (28.6%) of the 14 patients without liver metastases. Of the 32 patients who died of cancer-related causes, the 18 (56.3%) who suffered from liver metastases died within 30 months after surgery. On the other hand, the