(**Table 2**). Determination of this information prior to the operation will allow us to select the appropriate operative procedure or less invasive surgery.

In light of the above observations, controversy exists over how to treat early ampullary cancer, especially the use of ampullectomy. Some authors have advocated this procedure for T1 lesions, with preoperative endoscopic biopsy, EUS, and intraoperative frozen section as the procedures of choice to identify appropriate lesions. However, the reported sensitivity of preoperative biopsy is only 42% to 76% (17,23), which is not satisfactory preoperative diagnosis. Some authors recommend local resection, if technically possible, for small and likely benign tumors and for malignant tumors in patients at high operative risk. Then, close postoperative follow-up with duodenoscopy and ERCP is needed because of the risk of local recurrence. Hence, some surgeons propose that PD should be the procedure of choice in performing adequate radical resection, even in early ampullary cancer (24-28). Furthermore, some authors point out limitations of ampullectomy in recent years. The reduced morbidity and mortality of ampullectomy makes this the preferred treatment for benign lesions of the ampulla, but conversion to PD should be considered when intraoperative or final pathology identifies invasive adenocarcinoma (26,28,29). In short, ampullectomy should be used for benign tumor of the ampulla.

We experienced three cases of PHRSD, and all three patients are alive at this writing without recurrence. We have no direct evidence as to whether PHRSD can offer an operative cure of ampullary cancer, and long-term follow-up and further study are needed. In the present study, we examined the detailed clinicopathologic features of the tumors by reviewing specimens to evaluate the feasibility of less invasive surgery. If a patient is preoperatively diagnosed as Panc(-), Du(-) and N(-), less invasive surgery, like PHRSD, should be indicated. Taking into consideration the fact that PD should be performed for not only advanced cancer but also early cancer, PHRSD may well be a reasonable operative procedure. Because of the difficulty of accurate preoperative diagnosis of lymph node metastasis, however, debate may arise regarding whether PHRSD, PD or PpPD is the best treatment for early cancer.

In conclusion, we propose that PpPD and regional lymph node dissection are a reasonable surgical approach for ampullary cancer. In this study, there was no significant difference between the survival rates for PD and PpPD, and lymph node metastases were identified in 56.3% of all resected cases. This rate was much higher than we expected. Hence, regional lymph node dissection for ampullary cancer must be performed satisfactorily. On the other hand, ampullectomy should be used for benign tumor of the ampulla. If the tumor is preoperatively diagnosed as Panc(-), Du(-) and N(-), less invasive surgery is indicated, but we emphasize the importance of making an accurate diagnosis preoperatively.

## REFERENCES


1 **Monson JRT, Donohue JH, McEntee GP:** Radical resection for carcinoma of the ampulla of Vater. Arch Surg 1991; 126:353-357.
2 **Howe JR, Klimstra DS, Moccia RD:** Factors predictive of survival in ampullary carcinoma. Ann Surg 1998; 228:87-94.
3 **Nakao A, Harada A, Nonami T:** Prognosis of cancer of the duodenal papilla of Vater in relation to clinicopathological tumor extension. Hepatogastroenterology 1994; 41:73-78.
4 **Roder JD, Schneider PM, Stein HJ:** Number of lymph node metastases is significantly associated with survival in patients with radically resected carcinoma of the ampulla of Vater. Br J Surg 1995; 82:1693-1696.
5 **Allema JH, Reinders ME, van Gulik TM:** Results of pancreaticoduodenectomy for ampullary carcinoma and analysis of prognostic factors for survival. Surgery 1995; 117:247-253.
6 **Chareton B, Coiffic J, Landen S:** Diagnosis and therapy for ampullary tumors: 63 cases. World J Surg 1996; 20:707-712.
7 **Bottger TC, Boddin J, Heintz A:** Clinicopathologic study for the assessment of resection for ampullary carcinoma. World J Surg 1997; 21:379-383.
8 **Talamini MA, Moesinger RG, Pitt HA:** Adenocarcinoma of the ampulla of Vater. A 28-year experience. Ann Surg 1997; 225:590-599.
9 **Yeo CJ, Sohn TA, Cameron JL:** Periampullary adenocarcinoma: analysis of 5-year survivors. Ann Surg 1998; 227:821-831.
10 **Duffy JP, Hines OJ, Liu JH:** Improved survival for adenocarcinoma of the ampulla of Vater: fifty-five consecutive resections. Arch Surg 2003; 138:941-948.
11 **Park SJ, Kim SW, Jang JY:** Intraoperative transfusion: is it a real prognostic factor of periampullary cancer following pancreatoduodenectomy? World J Surg 2002; 26:487-492.
12 **Nakao A, Takeda S, Sakai M:** Extended radical resection versus standard resection for pancreatic cancer. Pancreas 2004; 28:289-292.
13 **Japanese Society of Biliary Surgery:** General Rules for Surgical and Pathological Studies on Cancer of Biliary Tract (4th Edition). Kanehara Shuppan: Tokyo, 1997.
14 **Nakao A:** Pancreatic head resection with segmental duodenectomy and preservation of gastroduodenal artery. Hepatogastroenterology 1998; 45:533-535.
15 **Chan C, Herrera MF, de la Garza L:** Clinical behavior and prognostic factors of periampullary adenocarcinoma. Ann Surg 1995; 222:632-637.
16 **Kayahara M, Nagakawa T, Ohta T:** Surgical strategy for carcinoma of the papilla of Vater on the basis of lymphatic spread and mode of recurrence. Surgery 1997; 121:611-617.
17 **Roberts RH, Krige JE, Bornman PC:** Pancreatoduodenectomy of ampullary carcinoma. Am Surg 1999; 65:1043-1048.
18 **Itoh A, Goto H, Naitoh Y:** Intraductal ultrasonography in diagnosing tumor extension of cancer of the papilla of Vater. Gastrointest Endosc 1997; 45:251-260.
19 **Cannon ME, Carpenter SL, Elta GH:** EUS compared with CT, magnetic resonance imaging, and angiography and the influence of biliary stenting on staging accuracy of ampullary neoplasms. Gastrointest Endosc 1999; 50:27-33.
20 **Shoup M, Hodel P, Aranha GV:** Defining a role for endoscopic ultrasound in staging periampullary tumors. Am J Surg 2000; 179:453-456.
21 **Harada N, Treitchke F, Imaizumi T:** Pancreatic invasion is a prognostic indicator after radical resection for carcinoma of the ampulla of Vater. J Hepatobiliary Pancreat Surg 1997; 4: 215-219.
22 **Bakkevold KE, Kambestad B:** Staging of carcinoma of the pancreas and ampulla of Vater: tumor (T), lymph node (N), and distant metastasis (M) as prognostic factors. Int J Pancreatol

1995; 17:249-259.

23 **Rattner DW, Fernandez-del Castillo C, Brugge WR:** Defining the criteria for local resection of ampullary neoplasms. Arch Surg 1996; 131:366-371.

24 **Branum GD, Pappas TN, Meyers WC:** The management of tumors of the ampulla of Vater by local resection. Ann Surg 1996; 224:621-627.

25 **Alstrup N, Burcharth F, Hauge C:** Transduodenal excision of tumours of the ampulla of Vater. Eur J Surg 1996; 162:961-967.

26 **de Castro SMM, van Heek NT, Kuhlmann KFD:** Surgical management of neoplasms of the ampulla of Vater: local resection or pancreatoduodenectomy and prognostic factors for survival. Surgery 2004; 136:994-1002.

27 **Dixon E, Vollmer Jr CM, Sahajpal A:** Transduodenal resection of peri-ampullary lesions. World J Surg 2005; 29:649-652.

28 **Yoon YS, Kim SW, Park SJ:** Clinicopathologic analysis of early ampullary cancers with a focus on the feasibility of ampullectomy. Ann Surg 2005; 242:92-100.

29 **Roggin KK, Yeh JJJ, Ferrone CR:** Limitations of ampullectomy in the treatment of nonfamilial ampullary neoplasms. Ann Surg Oncol 2005; 12:971-980.

# Clinical Experiment of Mutant Herpes Simplex Virus HF10 Therapy for Cancer

A. Nakao[1,*], S. Takeda[1], S. Shimoyama[1], H. Kasuya[1], H. Kimata[1], O. Teshigahara[1], M. Sawaki[1], T. Kikumori[1], Y. Kodera[1], T. Nagasaka[2], F. Goshima[3], Y. Nishiyama[3] and T. Imai[1]

[1]*Departments of Surgery II,* [2]*Histopathology and* [3]*Virology, Nagoya University, Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan*

**Abstract:** We reviewed our clinical trial using mutant herpes simplex virus "HF10". We have evaluated the safety and effect of HF10 against recurrent breast cancer since 2003 and also applied HF10 to non-resectable pancreatic cancer since 2005.

An oncolytic herpes simplex virus type 1, mutant HF10, has been isolated and evaluated for anti-tumor efficacy in syngeneic immunocompetent mouse models. From long time before clinical trial, we have found that the mutant virus can have remarkable potential to effectively treat cancer in experimental studies using animals, and that all of the surviving mice acquire resistance to rechallenge of the tumor cells. A number of studies have shown that HF10 is effective and safe for use in localized or peritoneally disseminated malignant tumors of non-neuronal origin in animals.

Pilot studies using HF10 have been initiated in patients with metastatic breast cancer. For each patient, 0.5 ml HF10 diluents at various doses were injected into test nodule, and 0.5 ml sterile saline was injected into a second nodule. All patients were monitored for local and systemic adverse effects, and the nodules were excised 14 days after viral injection for histopathological studies. All patients tolerated the clinical trial well. While no adverse effects occurred, there was cancer cell death and 30–100% regression histopathologically in recurrent breast cancer.

As mentioned above, intratumoral injection of mutant herpes simplex virus HF10 for recurrent metastatic breast cancer was safe and effective. Also a trial for non-resectable pancreatic cancer being carried out on the basis of the above result has proved to be innocuous and has been in progress to assess the clinical benefit and enhance the potentiality of HF10 against cancer.

**Keywords**: Oncolytic viral therapy, HF10, herpes virus therapy, breast cancer, pancreatic cancer, clinical experiment.

## INTRODUCTION

Although refinements have been made in multimodality therapies, such as novel chemotherapeutic and hormonal agents, they have been unable to prolong survival of patients, particularly those with advanced or recurrent breast cancer. Thus, breast cancer still poses a genuine threat to women. In another field of cancer, the prognosis for patients with pancreatic cancer is poor with or without treatment, and its early diagnosis remains difficult despite the development of new diagnostic modalities. Surgical resection maintains its role as the principle therapy for early-stage cancers and surgery combined with chemotherapy to provide the best hope for cure for patients suffering from malignant tumors. However, some new approaches are required to expand the possibilities for cure.

We established a syngeneic immunocompetent mouse model of disseminated peritoneal colon carcinoma to evaluate an oncolytic herpes simplex virus (HSV) type 1 mutant, HF10 [1-3], as a novel agent for cancer therapy. We earlier examined the feasibility of using HSV in the treatment of intraperitoneal dissemination of tumor and defined the biological determinants of its anti-tumor efficacy [4, 5]. The survival rate of the animals treated with a single intraperitoneal (i.p.) injection of $1 \times 10^7$ plaque forming units (pfu) of HF10 was 83.3%, while that of the control group treated with the same volume of saline was 33.3% [5]. The survival rate of the animals treated with 3 i.p. injections of $1 \times 10^7$ pfu of HF10 resulted in 100% survival, while that of the control group treated with the same amount of saline was 28.6% [5]. HSV antigen-positive cells were detected in a number of nodules, particularly around their degenerative and necrotic areas in the peritoneal cavity on day 5 [4, 5]. Other main organs such as liver, spleen, intestine, kidney, pancreas and brain were negative for HSV antigen-positive cells after treatment with HF10 [4, 5]. We also examined the effect of HF10 treatment of colon carcinomatosis in HSV-immunized mice and found that the immunized mice had 100% survival [5]. These results suggest that antibodies acquired against HF10 do not interfere with the therapeutic effect of viral treatment of colon carcinomatosis. Our data supports the potential use of HSV oncolytic therapy for humans with preexisting immunity to HSV. Our finding that mice rescued by HSV treatment were resistant to rechallenge with Colon26 cells [4, 5] indicates that the induction of an anti-tumor immune response may reduce tumor recurrence.

Moreover, the mouse breast cancer cell line, MM102-TC, and two human breast cancer cell lines, MCF-7 and YMB-1, were selected for *in vivo* flank tumor experiments in athymic nude mice. All of those cancer cells sensitive to HF10 showed tumor regression and prolonged survival [6]. Such antitumor effects are also observed in other malignant cells, including those of bladder cancers and sarcomas [4, 7].

We concluded that the efficacy of HF10 would depend on the extent of both intratumoral viral replication and induction of a host antitumor immune response, independent of the types of tumors. Since HF10 is replication-competent but highly attenuated, and its neurovirulence is highly decreased, side effects would occur very rarely. Even if some adverse events occurred, HF10 is hypersensitive to antiherpetic drugs such as acyclovir or ganciclovir, and thus can be safely administered to humans.

In this paper, we introduce a pilot study of intratumoral injection of HF10 into the subcutaneous nodules of recurrent breast cancer in six patients [8, 9] and a clinical experiment of

*Address correspondence to this author at the Department of Surgery II, Graduate School of Medicine, Nagoya University, 65 Tsurumai-cho, Showa-ku, Nagoya, 466-8550, Japan; Tel: +81 52 744 2232; Fax: +81 52 744 2255; E-mail: nakaoaki@med.nagoya-u.ac.jp

1568-0096/07 $50.00+.00

intratumoral injection of HF10 into the tumor of non-resectable pancreatic cancer in three patients.

## VIROLOGY OF HSV-1 STRAIN/ HF10

The HSV-1 strain, HF10, was a non-selected clone derived from our stock of HF. It is acknowledged to cause extensive cell membrane fusion in infected cells. The viruses were grown on embryonic chick cells and stored in aliquots at -80°C until use. Viral titers were assayed in embryo chick cells and expressed as plaque-forming units (PFU)/ml. The genome structure of HF10 is shown in Fig. (**1**), and its two main features may be summarized as follows: (i) A 3,832-base pair (bp) deletion between nucleotides 116,514 and 120,347 was detected, indicating that HF10 contained a complete open reading frame (ORF) of ICP 0 but had a partial deletion in the UL56 ORF and the complete loss of its promoter; and (ii) sequences between nucleotides 6,025 and 8,319 were deleted from the TRL, and the 6,027 bp DNA from nucleotide 110,488 up to 116-514 was inserted in an invested orientation. As a result, the HF10 contained two complete copies of UL53, UL54, and UL55, one complete and one partial copy of UL52, and two incomplete copies of UL56 without its promoter [4]. The role of the UL56 gene may be important to understand the molecular background of attenuation of HF10 *in vivo*. Our observations are consistent with previous reports [10, 11] that the lack of the UL56 product markedly reduces the neuroinvasiveness of HSV-1 without affecting viral replication in most types of cultured cells. HF10 was avirulent in mice after i.p. inoculation. We have recently shown [12] that the UL56 protein of HSV-2 is a tail-anchored type II membrane protein localizing in the Golgi apparatus and endosomes. The possibility that UL56 plays a role in vesicular trafficking points to its potential involvement in the anterograde axonal transport of viral envelope glycoproteins [12].

## CLINICAL EXPERIENCE

### Study Design

All six patients were female, aged 48 to 76, serum HSV antibody-positive, and diagnosed with breast cancer. While all of them had mastectomy, suffered a recurrence and underwent chemotherapy and/or endocrine therapy and/or radiation therapy, the recurrent foci were progressive, and metastasized to the cutaneous or subcutaneous region, which was pathologically proven to be recurrent breast cancer. The profiles of the patients are shown in Table **1**.

In this pilot study, we did a preliminary investigation of toxicity and possible efficacy in six patients with subcutaneous recurrent breast cancer to access the therapeutic potential of HF10 in human disease. We chose patients with metastatic skin cancers (lesions) so that the therapeutic virus could be injected subcutaneously.

For each patient, at least two tumor nodules were required, so that HF10 could be injected in one nodule and saline in another as a control. First, a test nodule around 1 cm in diameter was injected with HF10 diluent at various doses: Patient 1: 1 x $10^4$pfu/0.5 ml; patient 2: 1 x $10^5$pfu/0.5 ml; patient 3: 1 x $10^5$pfu/0.5 ml x 3 days; patient 4: 5 x $10^5$pfu/0.5 ml; and patients 5 and 6: 5 x $10^5$pfu/0.5 ml x 3 days. Then, the second nodule was injected with 0.5 ml sterile saline. All patients were monitored for local and systemic adverse effects, including body temperature, local heat and reddishness. The nodules were examined for size and inflammation. Dose escalations were shown in Table **1**. Blood tests were done to check CBC, HSV IgG, NK IL10, IL12, IFNα and IFNβ on days 0, 1, 3, 7, 10 and 21 after viral injection. The nodules were excised 14 days after viral injection for histopathological examinations by conventional hematoxylin-eosin (HE) staining and by immunofluorescence examination using anti-herpes simplex virus type 1 (DAKO Corporation, Glostrup, Denmark).

After completion of the HF10 pilot study for recurrent breast cancer, we started the same pilot study for non-resectable pancreatic cancer patients. The profiles of the non-resectable pancreatic cancer patients are shown in Table **2**. Before laparotomy these three patients were diagnosed as resectable, however, since peritoneal dissemination was found in Patient 7 and 8, and hepatic metastasis in patient 9 after laparotomy, they were considered non-resectable. HF10 diluent was directly injected during operation, and a small catheter was inserted into the tumor and fixed. The second and third injections were performed on postoperative first and second day *via* catheter. Blood tests were done the same as for breast cancer patients.

All patients gave written informed consent. This study was approved by the local ethics committee and by the institutional review board (IRB) of our hospital.

## THERAPEUTIC RESPONSE ASSESSMENT

### Evaluation of Safety and Effectiveness

In assessing the therapeutic response for breast cancer, we made use of the histopathological criteria for assessment of therapeutic response in breast cancer established by the

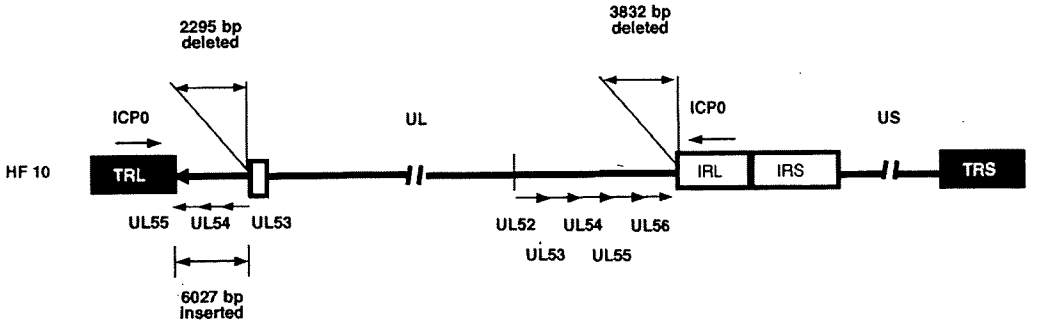


**Fig. (1).** A schematic representation of the structure of HF10. Locations of deletions and insertions in the genome of HF10 are shown. Derivations of the nucleotide positions are indicated in the text. An expansion indicates the position of genes within the deletion and insertion regions. Arrows indicate the position and orientation of genes with the expansions.

**Table 1. Recurrent Breast Cancer Patient Profiles**

| Patient no. | Age | Gender | Contents (pfu)/Times | Histologic response | Histopathology | Toxicity |
|---|---|---|---|---|---|---|
| 1 | 61 | Female | $1 \times 10^4$ /x 1 | Grade 1b | Invasive ductal carcinoma | (-) |
| 2 | 62 | Female | $1 \times 10^5$ /x 1 | Grade 1a | Invasive ductal carcinoma | (-) |
| 3 | 48 | Female | $1 \times 10^5$ /x 3 | Grade 2 | Invasive ductal carcinoma | (-) |
| 4 | 66 | Female | $5 \times 10^5$ /x 1 | Grade 1b | Invasive ductal carcinoma | (-) |
| 5 | 72 | Female | $5 \times 10^5$ /x 3 | Grade 2-3 | Mucinous carcinoma | (-) |
| 6 | 76 | Female | $5 \times 10^5$ /x 3 | Not applicable | Scirrhous carcinoma | (-) |

**Table 2. Non-Resectable Pancreatic Cancer Patient Profiles**

| Patient no. | Age | Gender | Contents (pfu)/Time | Histopathology | Toxicity |
|---|---|---|---|---|---|
| 7 | 46 | Male | $1 \times 10^5$ /x 3 | Invasive ductal carcinoma | (-) |
| 8 | 69 | Male | $1 \times 10^5$ /x 3 | Invasive ductal carcinoma | (-) |
| 9 | 61 | Male | $5 \times 10^5$ /x 3 | Invasive ductal carcinoma | (-) |

Committee for Production of Histopathological Criteria of the Japanese Breast Cancer Society [13] as shown in Table 3.

All six breast cancer patients tolerated the clinical trial well. While no adverse side effects occurred, we found cancer cell death and 30–100% regression histopathologically. In three pancreatic cancer patients, no adverse side effects were noted, but the antitumor effect has not been clarified yet.

### Macroscopic Findings

Macroscopically, the height of the recurrent nodule decreased in patient No. 3, who was injected with HF10 of $1 \times 10^5$pfu/0.5 ml x 3 days. In other patients, the macroscopic size of nodules did not show obvious regression. In three pancreatic cancer patients, CT revealed no change in the size of the pancreatic tumor during their hospital stay.

### Blood Tests

Local and systemic adverse events, including the toxicity of HF10 injection, were checked by the blood tests (WBC, HSV-DNA, HSVIgG, HSVIgM, etc.), body temperature and blood pressure, but no toxicities or side effects were observed. White blood cells, including HSV IgG, NK, IL10, IL12, IFNα and IFNβ, did not change. During their hospitalization, patients 7 and 9 showed no increase in CA19-9, whereas patient 8 did.

### Histopathology

Fig. (**2A**) is a photo of an excised saline injected nodule for hematoxylin-eosin (HE) staining from patient No. 1, revealing invasive ductal carcinoma with no cell death. Fig. (**2B**) is the tissue excised after 14 days of HF10 injection, showing a decrease in the number of malignant cells in the same patient. The inclusion bodies can be seen in the breast cancer cells (Fig. (**2C**)). Fig. (**2D**) is a photo of an excised saline-injected nodule for HE staining of patient No. 5. It shows mucinous carcinoma and malignant cells around the mucin. Fig. (**2E**) shows no malignant cells 14 days after HF10 injection in the same patient.

Overall there was about 30–100% malignant cell deaths or disappearances by HF10 injection, while no cell death was seen in the saline-injected nodules of patients Nos. 1 to 5 (Table **1**). In patient No. 6, because the cancer cells in the recurrent nodule were few, the efficacy of HF10 could not be determined.

**Table 3. Classification of Histologic Responses**

| | |
|---|---|
| Grade 0 | No response. Almost no change in cancer cells after treatment. |
| Grade 1 | Slight response.<br>1a → Mild response. Mild changes in cancer cells regardless of area, or marked changes in less than one-third of cancer cells.<br>1b → Moderate response. Marked changes in one-third or more, but less than two-thirds, of tumor cells. |
| Grade 2 | Marked response. Marked changes in two-thirds of tumor cells or more. |
| Grade 3 | Complete response.<br>Necrosis or disappearance of all tumor cells.<br>Replacement of all cancer cells by granuloma-like and/or fibrous tissue.<br>In cases with complete disappearance of cancer cells, pretreatment pathologic evidence of the presence of cancer is necessary. |

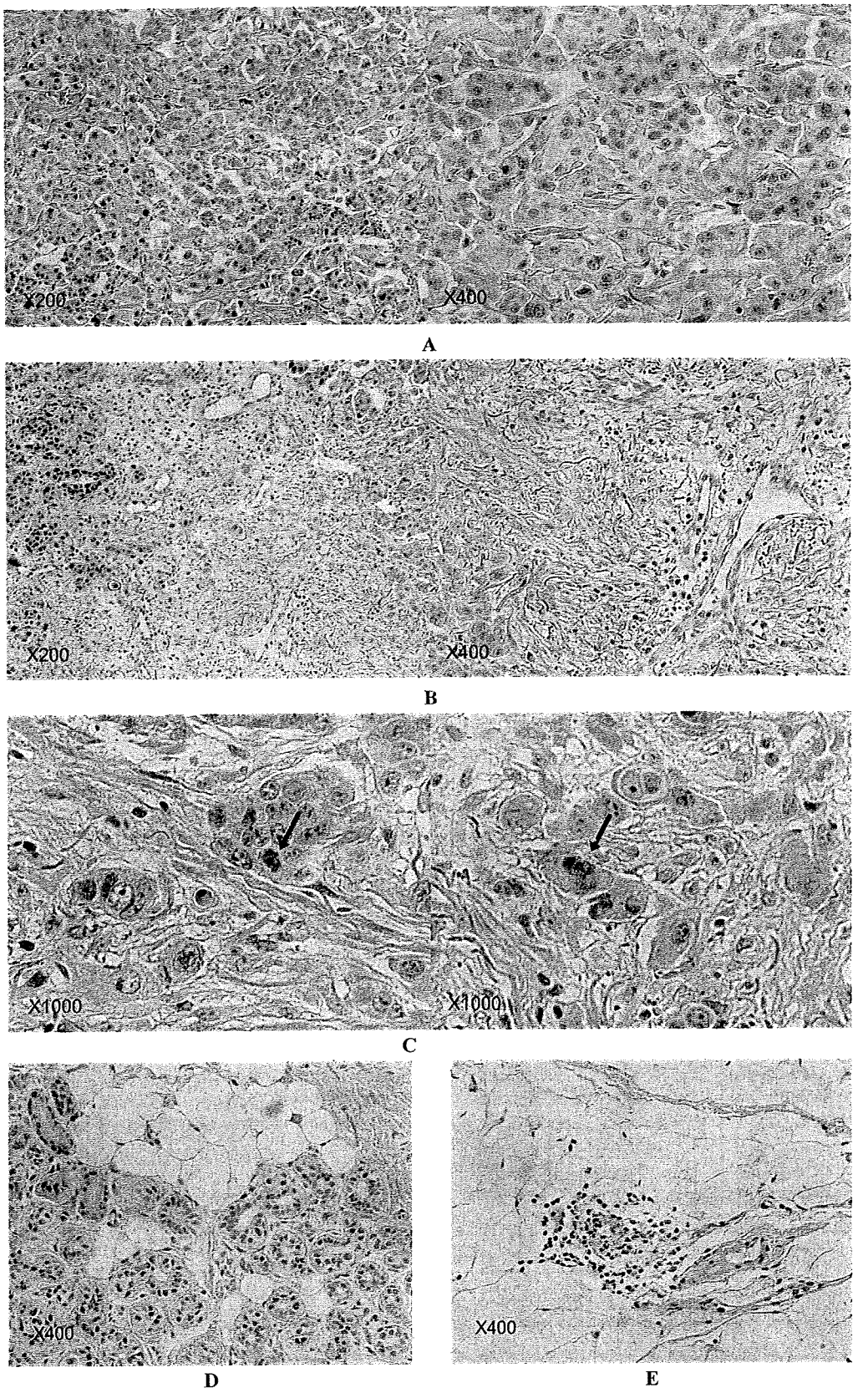


**Fig. (2).** Histological studies. *A* shows hematoxylin-eosin (H.E.) stained section of the saline-injected subcutaneous neoplasm of No. 1 patient. Histological studies reveal they are metastases of invasive ductal carcinoma of the breast. *B* shows the another lesion of the same patient after HF10 injection; the tumor area decreased about one thirds compared to *A*. *C* shows the inclusion body by arrow in the same patient. *D* shows hematoxylin-eosin (H.E.) stained section of the subcutaneous neoplasm of No. 5 patient. Histological studies reveal they are metastases of mucinous carcinoma of the breast. Tumor cells can be seen around the mucin. *E* shows the lesion of same patient after HF10 injection, whose tumor cells have totally disappeared in comparison with *D*.

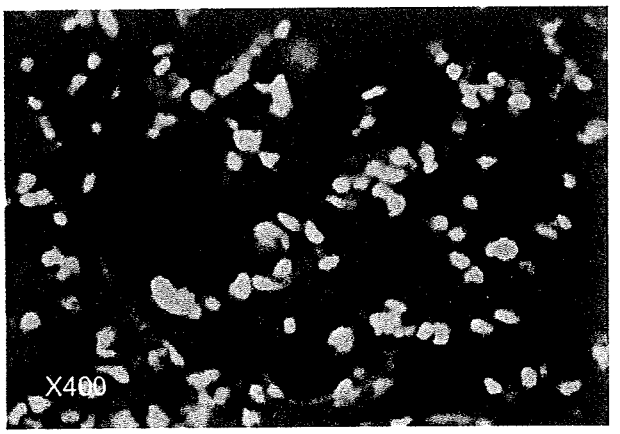


**Fig. (3).** Immunofluorescence examination. Immunofluorescence examination of the excised HF10-injected nodules showed evidence of viral infection confined to breast cancer cells in all patients.

### Immunofluorescence Examination

Immunofluorescence examination of the excised HF10 injected nodules revealed evidence of viral infection confined to the breast cancer cells (Fig. (3)) in all patients. Moreover, HF10 was seen in the breast cancer cells from the virus-treated nodules, with no antigen staining in the adjacent normal tissues of all six patients. The fact that each of the patients was seropositive for HSV before the virus injection indicates that the ability of HF10 to replicate within the tumor cells is not blocked by the previous exposure to HSV.

## CONCLUSION

Whole "oncolytic viral therapy" was originally derived from "gene therapy," and the term is now used for a novel, effective modality to defeat cancers by the extent of both the intratumoral replication and induction of the host anti-tumor immune response [14-17].

We have chosen HSV for this therapeutic virus, because it has the following useful and attractive characteristics: 1) HSV can infect almost all kinds of cells; 2) its infectious ability is higher than those of other viruses such as adenovirus and adeno-associated virus (AAV); 3) all basic sequences of the HSV genomes are known; 4) HSV can defeat the infected cells at low doses; and 5) antiviral agents against HSV are available such as ganciclovir and acyclovir.

Now, many HSV mutants have been engineered and evaluated for their therapeutic potential as agents in the treatment of cancers the world over. G207, NV1020 and MGH1 are examples [18-20]: G207 has deletions at both gamma 34.5 (RL1) loci and an insertion of *Escherichia coli* lacZ gene at UL39, while NV1020 has one deletion at both gamma 34.5 loci, a deletion of the internal repeated region and a loss of UL24. Both of them were made avirulent by inactivating gamma 34.5 loci, which we consider a gene necessary to exert anti-tumor potency efficiently. In fact, almost all clinical studies using NV1020 and G207 in the US have been discontinued because they are too attenuated to attack malignant tumors.

Thus, our group focused on other dispensable genes including UL56 for attenuation [11], maintaining the tumor killing ability, selected and purified HF10 from our stock of HSV-1 strain HF. HF10 has a deletion of 3829 bp in the right end of the UL and UL/IRL junction, which is inversely inserted into TRL, resulting in the loss of UL56 expression [5, 6]. Although the precise role of the gene product in the replication and pathogenicity of HSV is not well established, previous study [12] suggested that it is involved in vesicular trafficking in infected cells. From both *in vitro* and *in vivo* studies, HF10 has demonstrated marked anti-tumoral efficacy against many types of malignant tumors such as colon cancer, breast cancer, melanoma and sarcoma.

No adverse effects occurred in the patients treated with HF10, and cancer cell death and regression were 30–100% histopathologically in recurrent breast cancer. These data reveal HF10 to be a safe and effective anticancer agent. Oncolytic viral therapy using HF10 promises to become an effective new cancer therapy in the near future.

## ACKNOWLEDGEMENTS

This study was supported by Japan Society for the Promotion of Science (JSPS KAKENHI 16390358), a grant (04-23609) from the Princess Takamatsu Cancer Research Fund, the found from Takeda Printing Co., Ltd., AICA Kogyo Co., Ltd. and Netz Toyota Higashi Nagoya Co., Ltd.

## REFERENCES

[1] Nishiyama, Y.; Kimura, H.; Daikoku, T. Complementary lethal invasion of the central nervous system by nonneuroinvasive herpes simplex virus type 1 and 2. *J. Virol.* **1990**, *65*, 4520-4524.

[2] Yamada, Y.; Kimura, H.; Morishima, T.; Daikoku, T.; Maeno, K.; Nishiyama, Y. The pathogenicity of ribonucleotide reductase-null mutants of herpes simplex virus type 1 in mice. *J. Infect. Dis.* **1991**, *164*, 1091-1097.

[3] Nishiyama, Y.; Yamada, Y.; Kurachi, R.; Daikoku, T. Construction of a US3 lacZ insertion mutant of herpes simplex virus type 2 and characterization of its phenotype *in vitro* and *in vivo*. *Virology* **1992**, *190*, 256-268.

[4] Takakuwa, H.; Goshima, F.; Nozawa, N.; Yoshikawa, T.; Kimata, H.; Nakao, A.; Nawa, A.; Kurata, T.; Sata, T.; Nishiyama, Y. Oncolytic viral therapy using a spontaneously generated herpes simplex virus type 1 variant for disseminated peritoneal tumor in immunocompetent mice. *Arch. Virol.* **2003**, *148*, 813-825.

[5] Kimata, H.; Takakuwa, H.; Goshima, F.; Teshigahara, O.; Nakao, A.; Kurata, T.; Sata, T.; Nishiyama, Y. Effective treatment of disseminated peritoneal colon cancer with new replication-component herpes simplex viruses. *Hepato-Gastroenterol.* **2003**, *50*, 961-966.

[6] Teshigahara, O.; Goshima, F.; Takao, K.; Kohno, S.; Kimata, H.; Nakao, A.; Nishiyama, Y. Oncolytic viral therapy for breast cancer with Herpes Simplex Virus Type 1 mutant HF10. *J. Surg. Oncol.* **2004**, *85*, 42-47.

[7] Kohno, S.; Luo, C.; Goshima, F.; Nishiyama, Y.; Sata, T.; Ono, Y. Herpes simplex virus type 1 mutant HF10 oncolytic viral therapy for bladder cancer. *Urology* **2005**, *66*, 1116-1121.

[8] Nakao, A.; Kimata, H.; Imai, T.; Kikumori, T.; Teshigahara, O.; Nagasaka, T.; Goshima, F.; Nishiyama, Y. Intratumoral injection of herpes simplex virus HF10 in recurrent breast cancer. *Ann. Oncol.* **2004**, *15*, 987-991.

[9] Kimata, H.; Imai, T.; Kikumori, T.; Teshigahara, O.; Nagasaka, T.; Goshima, F.; Nishiyama, Y.; Nakao, A. Pilot study of oncolytic viral therapy using mutant herpes simplex virus (HF10) against recurrent metastatic breast cancer. *Ann. Surg. Oncol.* **2006**, *13*, 1078-1084.

[10] Becker, Y.; Hadar, J.; Tabor, E.; Ben-Hur, T.; Raibstein, I.; Rösen, A.; Darai, G. A sequence in Hpal-P fragment of herpes simplex virus-1 DNA determines intraperitoneal virulence in mice. *Virology* **1986**, *149*, 255-259.

[11] Berkowitz, C.; Moyal, M.; Rösen-Wolff, A.; Darai, G.; Becker, Y. Herpes simplex virus type 1 (HSV-1) UL56 gene is involved in viral intraperitoneal pathogenicity to immunocompetent mice. *Arch. Virol.* **1994**, *134*, 73-83.

[12] Koshizuka, T.; Goshima, F.; Takakuwa, H.; Nozawa, N.; Daikoku, T.; Koiwai, O.; Nishiyama, Y. Identification and characterization of the UL56 gene product of herpes simplex virus type 2. *J. Virol.* **2002**, *76*, 6718-6728.

[13] Committee for Production of Histopathological Criteria, Japanese Breast Cancer Society. Histopathological criteria for assessment of therapeutic response in breast cancer. *Breast Cancer* **2001**, *8*, 1-7.

[14] Culver, K. W.; Ram, Z.; Wallbridge, S.; Ishii, H.; Oldfield, E. H.; Blaese, R. M. *In vivo* gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors. *Science* **1992**, *256*, 1550-1552.

[15] Qazilbash, M. H.; Xiao, X.; Seth, P.; Cowan, K. H.; Walsh, C. E. Cancer gene therapy using a novel adeno-associated virus vector expressing human wild-type p53. *Gene Ther.* **1997**, *4*, 675-682.

[16] Martuza, R. L.; Malick, A.; Markert, J. M.; Ruffner, K. L.; Coen, D. M. Experimental therapy in human glioma by means of a genetically engineered virus mutant. *Science* **1991**, *252*, 854-856.

[17] Goldstein, D. J.; Weller, S. K. 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* **1988**, *166*, 41-51.

[18] Wong, R. J.; Kim, S. H.; Joe, J. K.; Shah, J. P.; Johnson, P. A.; Fong, Y. M. Effective treatment of head and neck squamous cell carcinoma by an oncolytic herpes simplex virus. *J. Am. Coll. Surg.* **2001**, *193*, 12-21.

[19] Cozzi, P. J.; Malhotra, S.; McAuliffe, P.; Kooby, D. A.; Federoff, H. J.; Huryk, B.; Johnson, P.; Scardino, P. T.; Heston, W. D. W.; Fong, Y. Intravesical oncolytic viral therapy using attenuated, replication-competent herpes simplex viruses G207 and Nv1020 is effective in the treatment of bladder cancer in an orthotopic syngeneic model. *FASEB J.* **2001**, *15*, 1306-1308.

[20] Meignier, B.; Martin, B.; Whitley, R. J.; Roizman, B. *In vivo* behavior of genetically engineered herpes simplex viruses R7017 and R7020. II. Studies in immunocompetent and immunosuppressed owl monkeys (Aotus trivirgatus). *J. Infect. Dis.* **1990**, *2*, 313-221.

**Received: July 05, 2006** **Revised: September 30, 2006** **Accepted: October 03, 2006**

World Journal of Surgery


Published Online: 30 November 2006

World J Surg (2007) 31: 147–154
DOI: 10.1007/s00268-005-0730-5

# Prognostic Implication of Para-aortic Lymph Node Metastasis in Resectable Pancreatic Cancer

Ryuichiro Doi, MD, Kazuhiro Kami, MD, Daisuke Ito, MD, Koji Fujimoto, MD, Yoshiya Kawaguchi, MD, Michihiko Wada, MD, Masafumi Kogire, MD, Ryo Hosotani, MD, Masayuki Imamura, MD, Shinji Uemoto, MD

*Department of Surgery, Kyoto University, 54 Shogoinkawaracho, Sakyo, Kyoto 606-8507, Japan*

**Abstract**

*Background:* The survival curve of patients who undergo surgical resection of pancreatic cancer displays a steep decline within 1 year and a relatively slow decline thereafter. The patients with a short survival time may have identifiable clinicopathologic factors that lead to rapid relapse.

*Study Design:* We analyzed clinicopathologic factors in 133 patients who underwent margin-negative pancreatoduodenectomy with extended radical lymphadenectomy for invasive ductal carcinoma of the pancreas to detect factors that could be responsible for the short survival.

*Results:* Tumor size, invasion of the anterior pancreatic capsule, retroperitoneal invasion, portal venous invasion, major arterial invasion, and metastasis to the para-aortic lymph nodes were variables associated with survival time in univariate analysis. Metastasis to the para-aortic lymph nodes was the single independent factor with a significant association with mortality in multivariate analysis. Some 84% of the patients who had positive para-aortic lymph nodes died within 1 year, versus 46% of the patients with negative nodes.

*Conclusions:* Although tumors that involve the para-aortic lymph nodes may technically be resectable, the expected postoperative survival time for most patients is less than 1 year. If para-aortic nodal metastasis is detected, alternative treatment strategies should be considered.

Pancreatic carcinoma is among the leading causes of cancer death. This tumor currently kills more than 17,000 persons per year in Japan, and is the fifth leading cause of cancer deaths.[1–3] The results of surgical treatment, including super-radical resection, are poor.[4,5] The overall 5-year survival rate for patients undergoing margin-negative resection is reported to be only 6.8%–25%.[6–11]

Since improvement of operative and perioperative management has reduced the operative mortality rate, most surgeons have maintained that resection provides the only hope of long-term survival for pancreatic cancer patients.[9,10,12] However, not all patients with systemic micro-metastases can be treated effectively by margin-negative surgical resection.[6–10] A percentage of these patients relapse soon after surgery, and their survival may be shorter than that of patients treated by systemic chemotherapy[13,14] or radiochemotherapy.[15–19] Pancreatic cancer seems to become a systemic disease beyond a certain stage of extrapancreatic spread, because extended radical lymph node dissection fails to prolong survival, even when the tumor is resectable.

We noted that the survival curve of patients who undergo surgical resection of pancreatic cancer exhibits a steep decline within 1 year and a relatively slow decline thereafter. The patients with a short survival time may have identifiable clinicopathologic factors that lead to early relapse of their disease. In this study, we analyzed the factors described in the classification system by the Japan Pancreas Society (JPS)[20] as well as those in the staging system by the TNM Committee of the Union

Correspondence to: Ryuichiro Doi, MD, e-mail: doir@kuhp.kyoto-u.ac.jp

International Contra la Cancrum (UICC)[21] or the American Joint Committee on Cancer (AJCC).[22] We hypothesized that pancreatic cancer with para-aortic lymph node involvement may represent systemic disease that fails to obtain any survival benefit from surgery. The objectives of the present study were to review our experience with margin-negative resection of pancreatic carcinoma and to evaluate the variables associated with survival.

## METHODS

Patients with histologically confirmed invasive ductal carcinoma of the head of the pancreas who had no clinical, radiographic, or intraoperative evidence of distant metastasis were reviewed. A series of 133 patients underwent pancreatoduodenectomy (PD) or pylorus-preserving pancreatoduodenectomy (PPPD) for pancreatic head cancer at Kyoto University Hospital between March 1980 and December 2000. All of these patients received histologically margin-negative resection with curative intent according to the definition given in the classification system published by the Japan Pancreas Society (JPS);[20] that is, no cancer cells were found within 5 ml of the resection lines in each direction.

Pancreatoduodenectomy was performed in an extended radical manner, and the regional lymph nodes belonging to group 1 (stations 13 and 17), group 2 (stations 6, 8, 12, and 14), and group 3 (stations 9, 11, 15, 16a2, 16b1, and 18) according to the fifth edition of the General Rules for the Study of Pancreatic Cancer issued by the Japan Pancreas Society (JPS 5th edition)[20] were routinely dissected. The para-aortic lymph nodes at stations 16a2 and 16b1[23] were also dissected from the upper part of the origin of the celiac trunk to the upper part of the origin of the inferior mesenteric artery. A semicircle of the nerve plexus at the root of the superior mesenteric artery was resected on the side closer to the tumor.

We examined the statistical significance of the factors defined in the JPS classification system[20] that were expected to affect the survival time. These factors included gender (male or female), age at surgery ( < 70 or > 70 years), tumor size (TS), invasion of the anterior pancreatic capsule (S), invasion of the retroperitoneal tissues (RP), invasion of the distal common bile duct (CH), invasion of the duodenal wall (DU), invasion of the portal venous system (PV), invasion of the major arteries (A), invasion of other organs (OO), invasion of the extrapancreatic plexus (PL), and lymph node metastasis (N). The level of each category was recorded on the basis of macroscopic intraoperative observations and microscopic pathological findings. In each patient, the microscopic findings received priority over the intraoperative findings. TNM staging was also done according to the sixth edition of the TNM staging system published by the Union International Contra la Cancrum (UICC, 6th edition)[21] or the American Joint Committee on Cancer (AJCC).[22] Furthermore, the effect of combined resection of the portal venous system (superior mesenteric vein or portal vein) and the influence of 5-fluorouracil (5-FU)-based adjuvant chemotherapy or adjuvant radiation therapy were assessed. Patients who received adjuvant radiation at doses greater than 24 Gy after surgical resection were classified as the radiotherapy group. The 60 patients in this group received an average radiation dose of 51.5 ± 2.3 Gy (mean ± SE; range: 25–87 Gy).

Categorized variables were analyzed by Fisher's exact test and the $\chi^2$ test. Survival rates after pancreatoduodenectomy were calculated by the Kaplan-Meier method,[24] and the differences between survival curves were assessed by the log-rank test. Testing of factors by univariate analysis was done as the first step. Then independent significant prognostic variables were identified by multivariate analysis using the Cox proportional hazards regression model. All statistical analyses were performed with JMP software (version 3.1.5, SAS Institute, Cary, NC) and StatView software (version J-4.5, Abacus Concepts, Berkeley, CA).

## RESULTS

The 133 patients were divided into two groups, one comprised of patients who survived for 12 months or less (n = 71) and a second made up of patients who were still alive at more than 12 months (n = 62). Clinical data were compared between the two groups (Table 1). The mean age was 62.8 ± 0.8 years (median: 64 years), and 62% of the patients were men. The age and gender distributions were not significantly different between the two groups.

The preoperative CA19-9 level was above 100 U/ml in 59 patients (64%). A total of 66 patients (50%) received adjuvant radiation therapy after curative resection, and the average dose was 51.5 ± 2.3 Gy (median: 50.4 Gy; range: 25–87 Gy). Seventy-one patients (54%) underwent combined resection of the portal venous system. Adjuvant radiation or venous resection was not a significant prognostic factor in our series.

Tumor factors defined by the JPS staging system (5th edition) are summarized in Table 2. Among these factors, tumor size (TS), invasion of the anterior pancreatic capsule

**Table 1.**
Comparison of the clinical characteristics of the 133 patients

| Variable | Subcategories | | Survival time < 12 months (n = 71) | Survival time > 12 months (n = 62) | *P* value |
|---|---|---|---|---|---|
| Gender | male | 83 (62%) | 47 | 36 | |
| | female | 50 (38%) | 24 | 26 | 0.37 |
| Age (years) | ≤70 | 105 (79%) | 54 | 51 | |
| | >70 | 28 (21%) | 17 | 11 | 0.40 |
| Pre-operative CA19-9 (U/ml)* | ≤100 | 33 (36%) | 12 | 21 | |
| | >100 | 59 (64%) | 32 | 27 | 0.13 |
| Vein resection # | No | 60 (46%) | 30 | 30 | |
| | Yes | 71 (54%) | 39 | 32 | 0.35 |
| Adjuvant radiation | No | 67 (50%) | 40 | 27 | |
| | Yes | 66 (50%) | 31 | 35 | 0.17 |
| Adjuvant chemotherapy | No | 57 | 31 | 26 | |
| | Yes | 75 | 39 | 36 | 0.86 |

* Pre-operative CA19-9 was not measured in some patients.
# Information for vein resection was not available in two patients.

**Table 2.**
Comparison of the tumor extension according to the General Rules for the Study of Pancreatic Cancer issued by the Japan Pancreas Society

| Variable (JPS) | subcategory | Survival time < 12 months (n = 71) | Survival time > 12 months (n = 62) | *P* value |
|---|---|---|---|---|
| Tumor size* | ≤2cm | 4 | 13 | |
| | 2cm<, ≤4cm | 30 | 29 | |
| | 4 cm<, ≤6cm | 24 | 14 | |
| | 6cm< | 12 | 4 | 0.01 |
| Invasion of the anterior pancreatic capsule (S) | S(−) | 45 | 52 | |
| | S(+) | 26 | 10 | 0.01 |
| Invasion of the retroperitoneal tissues (RP) | RP(−) | 35 | 44 | |
| | RP(+) | 36 | 18 | 0.01 |
| Invasion of the distal common bile duct (CH) | CH(−) | 24 | 25 | |
| | CH(+) | 47 | 37 | 0,48 |
| Invasion of the duodenal wall (DU) | DU(−) | 41 | 40 | |
| | DU(+) | 30 | 22 | 0.48 |
| Invasion of the portal venous system (PV) | PV(−) | 32 | 43 | |
| | PV(+) | 39 | 19 | 0.01 |
| Invasion of the major arteries (A) | A(−) | 57 | 55 | |
| | A(+) | 14 | 7 | 0.24 |
| Invasion of other organs (OO) | OO(−) | 52 | 58 | |
| | OO(+) | 19 | 4 | 0.001 |
| Invasion of the extrapancreatic plexus (PL)* | PL(−) | 18 | 30 | |
| | PL(+) | 18 | 11 | 0.06 |

IPS, Japan Pancreas Society.
* Informations for tumor size and plexus invasion were not available in some patients.

(S), retroperitoneal invasion (RP), portal venous invasion (PV), and invasion of other organs (OO) showed significant differences between the two groups in univariate analysis.

The TNM factors and the stage as defined by the JPS (5th edition) (Table 3), as well as the TNM factors and the stage as defined by UICC (6 edition) (Table 4), are shown. Lymph node metastasis to group 3 node is regarded as M1 by JPS staging system but M0 by UICC staging. All factors showed significant differences between the two groups.

When pancreatic fistula is defined by an amylase level in the drainage over 5,000 IU/l within 7 days of surgery, it

**Table 3.**
Comparison of the stage according to the General Rules for the Study of Pancreatic Cancer issued by the Japan Pancreas Society

| Category (JPS) | Sub category | Survival time < 12 months (n = 71) | Survival time > 12 months (n = 62) | *P* value |
|---|---|---|---|---|
| pT (JPS) | T1 | 1 | 8 | |
| | T2 | 3 | 4 | |
| | T3 | 18 | 22 | |
| | T4 | 49 | 28 | 0.01 |
| pN (JPS) | N0 | 19 | 30 | |
| | N1 | 21 | 15 | |
| | N2 | 15 | 14 | |
| | N3 | 16 | 3 | 0.01 |
| pM (JPS) | M0 | 55 | 59 | |
| | M1 | 16 | 3 | 0.01 |
| Stage (JPS) | 1 | 1 | 7 | |
| | 2 | 3 | 2 | |
| | 3 | 9 | 18 | |
| | 4a | 31 | 25 | |
| | 4b | 27 | 10 | 0.003 |

JPS, Japan Pancreas Society.

**Table 4.**
Comparison of the stage according to the TNM staging system by Union International Contra la Cancrum

| Category (UICC) | Sub category | Survival time < 12 months (n = 71) | Survival time > 12 months (n = 62) | *P* value |
|---|---|---|---|---|
| pT (UICC) | T1 | 3 | 14 | |
| | T2 | 1 | 0 | |
| | T3 | 40 | 37 | |
| | T4 | 27 | 11 | 0.001 |
| pN (UICC) | N0 | 19 | 30 | |
| | N1 | 52 | 32 | 0.01 |
| pM (UICC) | M0 | 71 | 62 | |
| | M1 | 0 | 0 | NA |
| stage (UICC) | La | 2 | 9 | |
| | 1b | 0 | 0 | |
| | 2a | 12 | 15 | |
| | 2b | 30 | 27 | |
| | 3 | 27 | 11 | |
| | 4 | 0 | 0 | 0.01 |

UICC, Union Internacional Contra la Cancrum.
NA, not applicable.

occurred in 5.6% (4/71) of the patients who survived for 12 months or less, and in 6.5% (4/62) of the patients who survived longer than 12 months. There was no significant difference between two groups with regard to the other complications.

Using our detailed records of the lymph node stations with metastasis, we compared the survival of patients with positive lymph nodes from group 1 (N1), group 2 (N2), and group 3 (N3) (Table 5). This comparison revealed that lymph node metastasis to group 3 had the most significant effect on the survival time.

Then we tested the patient factors and tumor factors for their relation to survival time by univariate analysis (Table 6). The results showed that tumor size (TS), invasion of the anterior pancreatic capsule (S), retroperitoneal invasion (RP), portal venous invasion (PV), major arterial invasion (A), and metastasis to group 3 lymph nodes (N3) were significantly associated with survival time. Therefore, these six variables were included in the multivariable analysis using the Cox proportional hazards regression model. As a result, we detected that lymph node metastasis involving group 3 nodes (N3) was the single independent factor with a significant effect on survival in our patient population (Table 7). Finally, these patients were categorized as N3, because all of them had a metastasized lymph node at station 16 (para-aortic lymph node).

To summarize the outcome of the multivariate analysis, the patients were divided into groups with or without metastasis to group 3 lymph nodes, and their survival curves were compared (Figure 1). The median survival time of the patients with metastasis to group 3 nodes was only 5.1 months (95% C.I. 3.9–9.2) and 84% of them died within 1 year. The median survival time of the comparison group was 12.4 months (95% C.I. 10.6–15.3), and 46% of them died within 1 year.

## DISCUSSION

Surgical resection is the only potentially curative therapy for pancreatic cancer. Unfortunately, because of the generally late presentation of this disease, only 15%–20% of patients are candidates for pancreatectomy.[1,25] The prognosis of pancreatic cancer is still poor in patients with potentially resectable disease, although there is some evidence that the outcome is improving over time.[26] As demonstrated in this study, there is a subgroup of surgically treated patients whose postoperative survival is shorter than 1 year. We believe that surgeons should avoid resection procedures in these patients because recently 1-year median survival time can be achieved by alternative treatments such as radiochemotherapy and systemic chemotherapy. Accordingly, we propose that positive para-aortic lymph node metastasis is one of the contraindications to surgical treatment.

**Table 5.**
Median survival times for 133 patients with various levels of lymph node metastasis

| Lymph node Metastasis | number of patients | Median survival time (months)* |
|---|---|---|
| N0 (node negative) | 49 | 14 |
| N1 (Group 1 positive) | 36 | 11.3 |
| N2 (Group 2 positive) | 29 | 10.6 |
| N3 (Group 3 positive) | 19 | 5.1 # |

* $P < 0.05$ by analysis of variance.
\# $P < 0.05$ versus NO by Tukey-Kramer test.

The prognosis of pancreatic cancer is poor, even in appropriately selected patients with negative surgical margins. Large series show 5-year survival rates of only 10%–25%, and the median survival time ranges between 10 and 20 months.[9,27–33] The most important prognostic factor for patients with complete resection is the node status. Five-year survival after pancreaticoduodenectomy has been reported to be only about 10% in patients who have node-positive disease, while it rises to 25% or 30% for those with node-negative disease.[9,28,31–33] Other reported predictors of a more favorable outcome include a tumor size of less than 3 cm, negative surgical margins, well-differentiated cancer, and intraoperative blood loss of less than 750 ml.[27–30,34]

In agreement with these reports, the current study showed that tumor size was a significant prognostic indicator by univariate analysis. Other factors detected in the current study were invasion of the anterior pancreatic capsule, invasion of retroperitoneal tissue, invasion of the portal venous system, invasion of major arteries, and lymph node metastasis. Chemotherapy with 5-FU as well as radiation therapy had no significant effect on survival time of the patients who received margin-negative resection, which is in harmony with our previous report.[35]

Recent reports showed that the number of positive nodes at any station is one of the important predicting factors for survival time of the surgically treated patient.[36,37] In addition to this finding, we finally demonstrated by multivariate analysis that para-aortic lymph node metastasis was an important factor associated with a shorter survival time. This is the first report to show that para-aortic lymph node metastasis affects the postoperative survival time of pancreatic cancer patients.

There is a consensus that disease limited to the pancreas and peripancreatic nodes is most likely to be cured by radical resection (stage I–IIB disease according to the UICC).[38] Absolute contraindications for resection include the presence of metastases to the liver, peritoneum, omentum, or any extra-abdominal site. Relative contraindications are involvement of the mesentery, the porto-mesenteric vessels, and the celiac axis (as well as its tributaries). If such contraindications are not found, the patient may be a candidate for radical resection. We believe that para-aortic lymph node metastasis should be included among the contraindications for curative resection in addition to the criteria mentioned above.

Although some authors have advocated palliative radical pancreatectomy for the management of pancreatic cancer, most surgeons would limit radical resection to patients with potentially curable lesions. This usually requires that the tumor does not involve any site that cannot be encompassed by the resection procedure and that it does not involve adjacent major vascular structures such as the superior mesenteric artery/vein, portal vein, celiac axis, or hepatic artery. In addition, the present findings suggest that we should avoid radical resection in patients with para-aortic lymph node metastasis. These patients are similar to those with metastases to the liver and other distant organs, i.e., they should be treated as having systemic disease.

It has been presumed that pancreatic cancer patients with para-aortic lymph node involvement may be better treated by methods other than curative resection. In the present study we reviewed data from patients who were treated by a consistent surgical method. In all of these patients, the para-aortic lymph nodes were routinely removed between the upper part of the origin of the celiac trunk and the upper part of the origin of the inferior mesenteric artery (stations 16a2 and 16b1).[23] The results of our analysis demonstrated that the survival time was likely to be shorter when para-aortic lymph node metastasis was present. Because the survival time of the patients with para-aortic lymph node metastasis was significantly short. Therefore, we think that intraoperative investigation of the para-aortic lymph node is very important.

Margin-negative resection should be a least requirement for cure of pancreatic cancer; however, we have shown that patients with para-aortic lymph node metastasis cannot be cured by margin-negative resection. Meanwhile some of the patients without para-aortic lymph node metastasis were cured by margin-negative resection. Therefore, we should perform radical resection to achieve margin-negative resection when the para-aortic lymph node metastasis is negative. On the other hand, if the patient is positive for para-aortic lymph node metastasis, we should not perform resection; instead, we should perform a bypass palliative procedure if needed.

It has been reported that prospective nodal staging of pancreatic carcinoma by computed tomographic (CT)

**Table 6.**
Univariate analysis of variables for mortality

| Variables | Subcategories | Hazard ratio (95% confidence intervals) | *P* value |
|---|---|---|---|
| Gender | male, female | 1.09 (0.90–1.32) | 0.39 |
| Age (years) | < 70, > 70 | 1.06 (0.65–1.64) | 0.81 |
| Vein resection | yes,- no | 1.09 (0.75–1.60) | 0.64 |
| Radiation | yes, no | 1.02 (0.70–1.47) | 0.93 |
| Chemotherapy | yes, no | 1.12 (0.77–1.63) | 0.57 |
| Preoperative CA19-9 (RJ/dL) | < 100, >100 | 1.32 (0.83–2.13) | 0.24 |
| Tumor size (cm) | <4, >4 | 1.55 (1.06–2.24) | **0.02** |
| Invasion of the anterior pancreatic capsule (S) | yes, no | 1.74 (1.14–2.60) | **0.01** |
| Invasion of the retroperitoneal tissues (RP) | yes, no | 1.49 (1:02–2.17) | **0.04** |
| Invasion of the distal common bile duct (CH) | yes, no | 1.24 (0.85–1.83) | 0.27 |
| Invasion of the duodenal wall (DU) | yes, no | 1.32 (0.90–1.93) | **0.16** |
| Invasion of the portal venous system (PV) | yes, no | 1.98 (1.35–2.91) | **0.0006** |
| Invasion of the major arteries (A) | yes, no | 1.83 (1.08–2.94) | **0.03** |
| Metastasis to Group 3 lymph node (N3) | yes, no | 3.21 (1.83–5.33) | **0.0001** |

**Table 7.**
Multivariable analysis of variables for survival

| | Hazard ratio (95% confidence intervals) | *P* value |
|---|---|---|
| Metastasis to Group 3 lymph node (N3) | 2.90 (1.60–5.02) | 0.001 |
| Invasion of the major arteries (A) | 1.52 (0.85–2.60) | 0.15 |
| Invasion of the portal venous system (PV) | 1.38 (0.84–2.24) | 0.20 |
| Invasion of the anterior pancreatic capsule (S) | 1.28 (0.77–2.09) | 0.33 |
| Tumor size | 1.04 (0.67–1.61) | 0.85 |
| Invasion of the retroperitoneal tissues (RP) | 1.02 (0.65–1.59) | 0.94 |

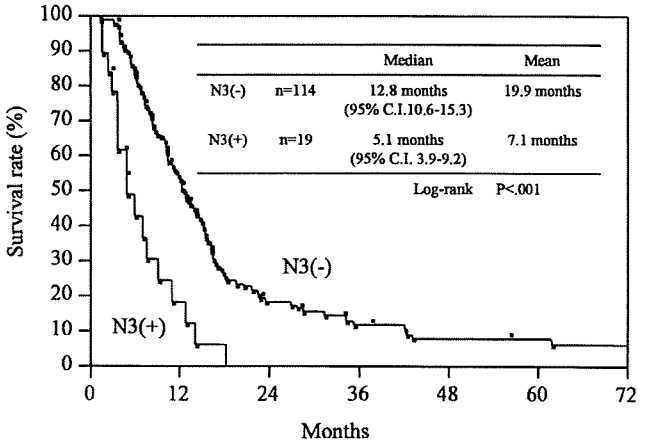


**Figure 1.** Survival of pancreatic cancer patients with or without metastasis to group 3 lymph node.

scanning is inaccurate.[39,40] Therefore, in a patient with a clinical diagnosis of pancreatic carcinoma that is considered to be otherwise resectable, the depiction on CT of enlarged peripancreatic or distant nodes should not be considered a contraindication to surgery. For these patients, endoscopic ultrasonography-guided lymph node biopsy should be tried to diagnose lymph node metastasis. When the biopsy is negative for metastasis or enlarged lymph nodes are not detected by CT and endoscopic ultrasonography, pancreatic resection is indicated; however, once intraoperative pathologic examination shows that nodes are positive, the treatment strategy should be revised on the basis of the pattern of lymph node metastasis.

We consistently performed extended lymphadenectomy as part of pancreatoduodenectomy, so this study was unable to evaluate the value of extended lymphadenectomy. The role of lymph node dissection in pancreatoduodenectomy has remained controversial for the last few decades.[41–44] There is no agreed definition of the extent of regional or extended lymphadenectomy for patients undergoing pancreatoduodenectomy. In the United States and Japan, however, this procedure most commonly involves removal of the peripancreatic lymph nodes as well as the soft tissues in the retroperitoneum from the hilum of the right kidney to the left lateral border

of the aorta along one axis and from the portal vein to the origin of the inferior mesenteric artery along the other axis.[41,45] Few studies have shown improved survival in patients who receive more extensive resection, including lymphadenectomy, and these studies have been performed on small populations.[41] In contrast, prospective randomized trials have failed to support the extent of lymphadenectomy as a significant prognostic factor.[42–44] At present, the available data suggest that para-aortic nodal metastases are a marker of systemic disease and that their removal is unlikely to alter overall survival. In addition, we have observed similar survival data in patients with para-aortic lymph node metastasis who had not undergone surgical resection but who received systemic chemotherapy or radiochemotherapy. Our results indicate that positive para-aortic nodes should lead to treatments other than surgical resection. However, in future, it still should be considered as a possible option for palliation, particularly when the effectiveness of adjuvant therapy improves. It also should be acknowledged that although the results of this study demonstrated that positive para-aortic lymph nodes are associated with a higher risk of mortality, the results should be verified in the individual patient before any decision is made to alter patient care.

In conclusion, we consider that intraoperative sampling of the lymph nodes at station 16 should be done before other surgical procedures. Although tumors involving the para-aortic lymph nodes may be technically resectable, the expected postoperative survival time is less than 1 year. If para-aortic nodal metastasis is detected, the current results should be considered in choosing between resection or treatment by radiochemotherapy or systemic chemotherapy.

## ACKNOWLEDGMENT

This work was supported by a grant-in-aid (No. 17390364) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

## REFERENCES

1. Matsuno S, Egawa S, Fukuyama S, *et al.* Pancreatic Cancer Registry in Japan: 20 years of experience. Pancreas 2004;28:219–230.
2. Yamamoto M, Ohashi O, Saitoh Y. Japan Pancreatic Cancer Registry: current status. Pancreas 1998;16:238–242.
3. Edge SB, Schmieg RE Jr, Rosenlof LK, *et al.* Pancreas cancer resection outcome in American university centers in 1989–1990. Cancer 1993;71:3502–3508.
4. Imaizumi T, Hanyu F, Harada N, *et al.* Extended radical Whipple resection for cancer of the pancreatic head: operative procedure and results. Dig Surg 1998;15:299–307.
5. Nagakawa T, Nagamori M, Futakami F, *et al.* Results of extensive surgery for pancreatic carcinoma. Cancer 1996; 77:640–645.
6. Niederhuber JE, Brennan MF, Menck HR. The National Cancer Data Base report on pancreatic cancer. Cancer 1995;76:1671–1677.
7. 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.
8. Livingston EH, Welton ML, Reber HA. Surgical treatment of pancreatic cancer. The United States experience. Int J Pancreatol 1991;9:153–157.
9. Trede M, Schwall G, Saeger HD. Survival after pancreatoduodenectomy. 118 consecutive resections without an operative mortality. Ann Surg 1990;211:447–458.
10. Cameron JL, Pitt HA, Yeo CJ, *et al.* One hundred and forty-five consecutive pancreaticoduodenectomies without mortality. Ann Surg 1993;217:430–435.
11. Cress RD, Yin D, Clarke L, *et al.* Survival among patients with adenocarcinoma of the pancreas: a population-based study (United States). Cancer Causes Control 2006; 17:403–409.
12. Schafer M, Mullhaupt B, Clavien PA. Evidence-based pancreatic head resection for pancreatic cancer and chronic pancreatitis. Ann Surg 2002;236:137–148.
13. Burris HA 3rd, Moore MJ, Andersen J, *et al.* Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. J Clin Oncol 1997;15:2403–2413.
14. Louvet C, Labianca R, Hammel P, *et al.* Gemcitabine in combination with oxaliplatin compared with gemcitabine alone in locally advanced or metastatic pancreatic cancer: results of a GERCOR and GISCAD phase III trial. J Clin Oncol 2005;23:3509–3516.
15. Crane CH, Janjan NA, Evans DB, *et al.* Toxicity and efficacy of concurrent gemcitabine and radiotherapy for locally advanced pancreatic cancer. Int J Pancreatol 2001;29:9–18.
16. Epelbaum R, Rosenblatt E, Nasrallah S, *et al.* Phase II study of gemcitabine combined with radiation therapy in patients with localized, unresectable pancreatic cancer. J Surg Oncol 2002;81:138–143.
17. de Lange SM, van Groeningen CJ, Meijer OW, *et al.* Gemcitabine-radiotherapy in patients with locally advanced pancreatic cancer. Eur J Cancer 2002;38:1212–1217.
18. Okusaka T, Ito Y, Ueno H, *et al.* Phase II study of radiotherapy combined with gemcitabine for locally advanced pancreatic cancer. Br J Cancer 2004;91:673–677.
19. Oya N, Shibuya K, Sakamoto T, *et al.* Chemoradiotherapy in patients with pancreatic carcinoma: phase-I study with a

fixed radiation dose and escalating doses of weekly gemcitabine. Pancreatology 2005;6:109–116.
20. Japan Pancreas Society. Classification of pancreatic carcinoma (2nd English edition). Japan Pancreas Society, editor, Tokyo, Kanehara & Co., 2003.
21. TNM Classification of Malignant Tumours, 6th ed. Sobin LH, Wittekind C, editors, New York, Wiley-Liss, 2002.
22. The American Joint Committee on Cancer. AJCC cancer staging manual, 6th ed, Greene FL, Page DL, Fleming ID, editors, New York, Springer-Verlag, 2002.
23. Committee on Classification of Regional Lymph Nodes of Japan Society of Clinical Oncology. Classification of regional lymph nodes in Japan. Int J Clin Oncol 2003;8:248–275.
24. Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. J Am Stat Assoc 1958;53:457–481.
25. Sener SF, Fremgen A, Menck HR, *et al.* Pancreatic cancer: a report of treatment and survival trends for 100,313 patients diagnosed from 1985–1995, using the National Cancer Database. J Am Coll Surg 1999;189:1–7.
26. Neoptolemos JP, Stocken DD, Friess H, *et al.* A randomized trial of chemoradiotherapy and chemotherapy after resection of pancreatic cancer. N Engl J Med 2004; 350:1200–1210.
27. Sohn TA, Lillemoe KD, Cameron JL, *et al.* Adenocarcinoma of the duodenum: factors influencing long-term survival. J Gastrointest Surg 1998;2:79–87.
28. Geer RJ, Brennan MF. Prognostic indicators for survival after resection of pancreatic adenocarcinoma. Am J Surg 1993;165:68–72; discussion 72–63.
29. Benassai G, Mastrorilli M, Quarto G, *et al.* Survival after pancreaticoduodenectomy for ductal adenocarcinoma of the head of the pancreas. Chir Ital 2000;52:263–270.
30. Millikan KW, Deziel DJ, Silverstein JC, *et al.* Prognostic factors associated with resectable adenocarcinoma of the head of the pancreas. Am Surg 1999;65:618–623.
31. Bakkevold KE, Arnesjo B, Dahl O, *et al.* Adjuvant combination chemotherapy (AMF) following radical resection of carcinoma of the pancreas and papilla of Vater—results of a controlled, prospective, randomised multicentre study. Eur J Cancer 1993;29A:698–703.
32. Tsao JI, Rossi RL, Lowell JA. Pylorus-preserving pancreatoduodenectomy. Is it an adequate cancer operation. Arch Surg 1994;129:405–412.
33. Yeo CJ, Cameron JL, Lillemoe KD, *et al.* Pancreaticoduodenectomy for cancer of the head of the pancreas. 201 patients. Ann Surg 1995;221:721–731.
34. Meyer W, Jurowich C, Reichel M, *et al.* Pathomorphological and histological prognostic factors in curatively resected ductal adenocarcinoma of the pancreas. Surg Today 2000; 30:582–587.
35. Nishimura Y, Hosotani R, Shibamoto Y, *et al.* External and intraoperative radiotherapy for resectable and unresectable pancreatic cancer: analysis of survival rates and complications. Int J Radiat Oncol Biol Phys 1997;39:39–49.
36. 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.
37. Ferrone CR, Kattan MW, Tomlinson JS, *et al.* Validation of a postresection pancreatic adenocarcinoma nomogram for disease-specific survival. J Clin Oncol 2005;23:7529–7535.
38. DiMagno EP, Reber HA, Tempero MA. AGA technical review on the epidemiology, diagnosis, and treatment of pancreatic ductal adenocarcinoma. American Gastroenterological Association. Gastroenterology 1999;117:1464–1484.
39. Roche CJ, Hughes ML, Garvey CJ, *et al.* CT and pathologic assessment of prospective nodal staging in patients with ductal adenocarcinoma of the head of the pancreas. AJR Am J Roentgenol 2003;180:475–480.
40. Imamura M, Doi R, Imaizumi T, *et al.* A randomized multicenter trial comparing resection and radiochemotherapy for resectable locally invasive pancreatic cancer. Surgery 2004;136:1003–1011.
41. 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.
42. 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: a multicenter, prospective, randomized study. Lymphadenectomy Study Group. Ann Surg 1998;228:508–517.
43. Yeo CJ, Cameron JL, Sohn TA, *et al.* Pancreaticoduodenectomy with or without extended retroperitoneal lymphadenectomy for periampullary adenocarcinoma: comparison of morbidity and mortality and short-term outcome. Ann Surg 1999;229:613–622; discussion 622–614.
44. Yeo CJ, Cameron JL, Lillemoe 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.
45. Pisters P, Brennan M. Regional lymph node dissection for pancreatic adenocarcinoma. In D Evans P Pisters J Abbruzzese editors, Pancreatic Cancer. New York, Springer-Verlag, 2002, pp 139–151.

Journal of Surgical Research 142, 281–286 (2007)
doi:10.1016/j.jss.2007.03.068

# Effect of the XIAP Inhibitor Embelin on TRAIL-Induced Apoptosis of Pancreatic Cancer Cells

Tomohiko Mori, M.D., Ryuichiro Doi, M.D.,[1] Atsushi Kida, M.D., Kazuyuki Nagai, M.D., Kazuhiro Kami, M.D., Daisuke Ito, M.D., Eiji Toyoda, M.D., Yoshiya Kawaguchi, M.D., and Shinji Uemoto, M.D.

*Department of Surgery, Division of Hepato-Biliary-Pancreatic Surgery and Transplantation, Kyoto University, Kyoto, Japan*

Submitted for publication January 8, 2007

***Background.*** **Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a potent inducer of apoptosis in a wide variety of tumor cells, while it has no toxicity for the majority of normal cells.Therefore, TRAIL may be a suitable agent for anticancer therapy. We previously reported that a number of pancreatic cancer cell lines show resistance to TRAIL-induced apoptosis via overexpression of XIAP and FLIP. The present study was conducted to further examine TRAIL-based therapeutic strategies by aiming to restore functional apoptotic pathways in resistant pancreatic cancer cells.**

***Methods.*** **In various pancreatic cancer cell lines, TRAIL-induced apoptosis was evaluated in the presence or absence of an XIAP-inhibitor (Smac peptide). Second, TRAIL-induced apoptosis was evaluated in TRAIL-resistant AsPC-1 cells with or without FLIP antisense. Third, the combined effect of Smac peptide and FLIP antisense was tested, and the activation of apoptosis-related caspases and poly (ADP-ribose) polymerase was evaluated. Finally, TRAIL-induced apoptosis was evaluated in the presence or absence of FLIP antisense and an XIAP inhibitor (embelin).**

***Results.*** **Smac peptide enhanced TRAIL-induced apoptosis in a dose-dependent manner for several pancreatic cancer cell lines, but showed no effect on TRAIL-resistant AsPC-1 cells. Smac peptide alone had no influence on cell viability. TRAIL-induced apoptosis was restored in TRAIL-resistant AsPC-1 cells by exposure to FLIP antisense, which suppressed the expression of FLIP. The effect of TRAIL was augmented by the combination of FLIP antisense and Smac peptide. Similarly, TRAIL-induced apoptosis was restored by the combination of FLIP antisense and embelin. Activation of apoptotic caspases and cleavage of poly (ADP-ribose) polymerase was observed after sensitization of TRAIL-resistant pancreatic cancer cells.**

***Conclusions.*** **Pancreatic cancer cells gain resistance to TRAIL-induced apoptosis via expression of the antiapoptotic proteins XIAP and FLIP. Smac peptide and FLIP antisense could restore the apoptotic effect of TRAIL. An XIAP inhibitor, embelin, enhanced the effect of TRAIL in the presence of FLIP antisense. These findings may provide useful information for the development of TRAIL-based therapeutic strategies by restoring functional apoptotic pathways in resistant pancreatic cancer cells. In addition, a low molecular weight XIAP inhibitor like embelin could be a lead compound for the development of effective XIAP inhibitors.** 

***Key Words:*** **apoptosis; caspase; Smac; pancreatic neoplasm; XIAP.**

## INTRODUCTION

Pancreatic cancer is one of the most highly malignant tumors and patients have a very low survival rate. The majority of patients diagnosed with pancreatic cancer present when their tumors are already unresectable and frequently need chemotherapy and radiotherapy. One of the reasons for their low survival rate is the poor response of pancreatic cancer to most current anticancer treatments such as chemotherapy and radiotherapy. The development and progression of this cancer, as well as resistance to most current therapies, are mainly related to a lack of response to apoptotic stimuli. Therefore, novel therapeutic strategies that overcome the resistance of pancreatic cancer to apoptosis would be useful to improve the survival of patients with this disease.

[1] To whom correspondence and reprint requests should be addressed at Department of Surgery, Division of Hepato-Biliary-Pancreatic Surgery and Transplantation, Kyoto University, 54 Shogoin Kawaracho, Sakyo-ku, Kyoto, 606-8507, Japan. E-mail: doir@kuhp.kyoto-u.ac.jp.



0022-4804/07 $32.00

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), also known as Apo-2 ligand, is a member of the tumor necrosis factor (TNF) family that preferentially triggers apoptosis in various tumor cells via two death domain-containing agonistic receptors, DR4 and DR5. Both DR4 and DR5 contain a cytoplasmic death domain that is required for TRAIL receptor-induced apoptosis [1, 2]. TRAIL also binds to DcR1 and DcR2, which act as decoy receptors that inhibit TRAIL signaling. Unlike DR4 and DR5, DcR1 does not have a cytoplasmic domain, while DcR2 only possesses a cytoplasmic fragment containing a truncated form of the consensus death domain motif [1].

The TRAIL pathway represents a potentially promising target for anticancer therapy. However, there are several endogenous intracellular proteins that could inhibit TRAIL-induced apoptosis [1, 2]. Although TRAIL might be an effective anticancer treatment, the mechanism of resistance to TRAIL-induced apoptosis needs to be clarified before its clinical use for pancreatic cancer.

We have previously reported that treatment with cycloheximide could enhance and restore apoptosis in TRAIL-resistant cells, as well as caspase-8 activation [3]. Such findings suggested that the death machinery was intact and functional, but possibly under negative regulation by antiapoptotic proteins, the expression of which was inhibited by cycloheximide. We have also reported that the expression of FLIP and XIAP in TRAIL-resistant cells was clearly suppressed by cycloheximide. Thus, it is possible that these proteins are major inhibitors of TRAIL-induced apoptosis, so that their modulation could restore the efficacy of TRAIL-based treatment for pancreatic cancer.

Accordingly, the present study was conducted to examine therapeutic strategies aimed at restoring the function of the apoptotic pathways in pancreatic cancer cells. For this purpose, we used FLIP antisense, a synthetic Smac peptide containing the four N-terminal residues essential for inactivation of XIAP, and an XIAP inhibitor (embelin, a small molecule derived from the Japanese herb Ardisia) to modulate the apoptotic pathways.

## MATERIALS AND METHODS

### Cell Lines and Culture

Five human pancreatic cancer cell lines (CFPAC-1, AsPC-1, PANC-1, BxPC-3, and Suit-2) were cultured in the following media at 37°C under a humidified atmosphere of 5% $CO_2$/95% air. CFPAC-1 cells were cultured in Iscove's modified Dulbecco's medium (Invitrogen Japan K.K., Tokyo, Japan) with 10% fetal bovine serum (FBS), PANC-1, Suit-2 cells were grown in DMEM with 10% FBS, and BxPC-3, and AsPC-1 cells were incubated in RPMI 1640 medium with 10% FBS. Each medium contained 100 U/mL penicillin and 100 μg/mL streptomycin.

### Antibodies, Recombinant Proteins, and Other Reagents

The following antibodies, recombinant proteins, and other reagents were purchased from the indicated sources: Rabbit anticaspase-9 antibody and rabbit anticaspase-3 (Cell Signaling Technology, Beverly, MA), mouse anti-FLIP antibody (Apotech Corporation, Lausen, Switzerland), mouse anti-XIAP antibody (MBL, Nagoya, Japan), rabbit anticaspase-8 antibody and mouse anti-poly (ADP-ribose) polymerase (PARP) antibody (BD PharMingen, San Diego, CA), mouse β-actin antibody (Sigma Chemical Co., St. Louis, MO), and recombinant human TRAIL (Pepro Tech EC Ltd., London, United Kingdom).

### Western Blot Analysis

Cells were lysed in ice-cold RIPA buffer (10 mM PBS, pH 7.4, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 5 mM ethylenediaminetetraacetic acid) supplemented with 1% phenylmethyl-sulfonyl fluoride and 20 μg/mL gabexate mesilate (FOY; Ono Pharmaceutical, Osaka, Japan) for 30 min at 4°C. Then the lysate was homogenized and centrifuged at 15,000 rpm for 30 min at 4°C to remove debris, and the protein concentration was measured using a BCA protein assay kit (Pierce, Rockford, IL). Equal amounts of protein were loaded onto SDS- polyacrylamide gels (8% to 12%) and the proteins were transferred to polyvinylidene difluoride membranes (IPVH304F0; Millipore, Billerica, MA). The blots were blocked overnight at 4°C with 5% (wt/vol) skim milk in TBST buffer (10 mM Tris-HCl, 150 mM NaCl, and 0.5% Tween-20). Subsequently, immunoblotting was done overnight at 4°C with an appropriate primary antibody. Excess antibody was removed by washing the membrane with TBST (3 times for 10 min each). The membrane was then incubated with a horseradish peroxidase- or alkaline phosphatase-conjugated secondary antibody for 1 h at room temperature, followed by the addition of TBST as described above. Reaction products were visualized by using ECL Western Blotting Detection Reagent (Amersham, Buckinghamshire, United Kingdom) or by using alkaline phosphatase solution supplemented with 100 mM Tris-HCl, 100 mM HCl, 5 mM $MgCl_2$, 0.03% nitroblue tetrazolium and 0.017% 5-bromo-indolylphosphate P-toluidine salt.

### Cell Viability Assay

Cells were seeded at a density of 5000 per well into 96-well plates in culture medium containing 10% FBS. After 24 h, the cultures were washed with fresh medium and treated with the indicated agents. After another 24 h, the number of viable cells was counted by using a Cell Counter Kit 8 (Dojindo Co., Kumamoto, Japan) according to the manufacturer's instructions. The assay reagent was a tetrazolium compound (WST-8) that was reduced by viable cells to yield a colored formazan product (detected at 450 nm). The amount of this formazan product was directly proportional to the number of viable cells in each culture.

### Peptide Design and Synthesis

On the basis of the fact that arginine-rich peptides show efficient translocation across cell membranes, the seven N-terminal residues of the Smac peptide were conjugated with eight arginine residues (AVPIAQK-GGRRRRRRRRGC), and a reversed version of Smac was conjugated with arginine repeats (KQAIPVA-GGRRRRRRRRGC) as a control peptide. These peptides were synthesized by Funakoshi, Inc. (Tokyo, Japan) for use in the present study.

### Delivery of Antisense PMO into Cultured Cells

Phosphorodiamidatemorpholino oligomer (PMO) for FLIP antisense and a standard control oligomer (control PMO) were produced by Gene Tools, Inc. (St. Louis, MO) with the following sequences: FLIP antisense PMO, 5′ATGACTTCAGCAGACATCCTACTCT3′; and standard control PMO, 5′CCTCTTACCTCAGTTACAATTTATA3′.

The control was designed to have no target and no significant biological activity, except in reticulocytes from humans with thalassemia who have a splice-generating mutation at position 705 in β-globin pre-mRNA. The delivery procedure was performed according to the recommendations of Gene Tools. The delivery formulation consisted of a prepared duplex of PMO and partially complimentary DNA oligomer together with a weakly basic delivery reagent (ethoxylated polyethylenimine). Because the morpholino oligomers are stable and nuclease resistant, there was no need to repeat the delivery procedure.

AsPC-1 cells were treated with FLIP antisense PMO and control PMO for 2 d. The delivery mixture was an aqueous solution of 0.5 mM FLIP antisense PMO or 0.5 mM control PMO and morpholino/DNA stock solution (Gene Tools). To this was added 200 μM ethoxylated polyethylenimine special delivery solution, followed by vortexing and incubation at room temperature for 20 min to generate the complete delivery solution. Then the medium was removed and the solution was added to cells, which were placed into a $CO_2$ incubator. After 3 h, the delivery solution was aspirated and replaced with fresh serum-containing medium.

### Statistical Analysis

Results are presented as the mean ± SD. Data were analyzed by Student's *t*-test, and statistical significance was accepted at a *P*-value of less than 0.05. Differences among three groups were assessed by analysis of variance, followed by a post hoc Tukey-Kramer test when appropriate. Each experiment was repeated independently at least three times.

## RESULTS

### Effect of Smac Peptide on TRAIL-Induced Apoptosis of Pancreatic Cancer Cells

The effect of Smac peptide on TRAIL-induced apoptosis was determined for various pancreatic cancer cell lines. We investigated whether this cell-permeable synthetic peptide containing the four N-terminal residues essential for XIAP inactivation could induce apoptosis, while the control peptide was a reversed version of Smac bound to arginine repeats.

Treatment with TRAIL in the presence of Smac peptide resulted in a significant increase of cell death in a dose-dependent manner for the Suit-2, CFPAC-1, Panc-1, and BxPC-3 cell lines (Fig. 1). However, TRAIL had no effect on TRAIL-resistant AsPC-1 cells, which strongly overexpress FLIP-S, even in the presence of Smac peptide.

### Effect of FLIP Antisense on TRAIL-Induced Apoptosis of AsPC-1 Cells

Next, we tested whether direct down-regulation of FLIP by FLIP antisense could restore TRAIL sensitivity to TRAIL-resistant AsPC-1 cells. Cells were treated with FLIP antisense or control oligonucleotides and subsequently exposed to various concentrations of TRAIL. We found that treatment with FLIP antisense could suppress the expression of FLIP-L and FLIP-S in AsPC-1 cells (Fig. 2A). In addition, TRAIL induced cell death in the presence of FLIP antisense, although the effect on cell viability was relatively modest (Fig. 2B).

### Effect of Combined Use of FLIP Antisense and Smac Peptide on TRAIL-Induced Apoptosis of AsPC-1 Cells

To modulate the TRAIL resistance of AsPC-1 cells, we tested the combination FLIP antisense and Smac

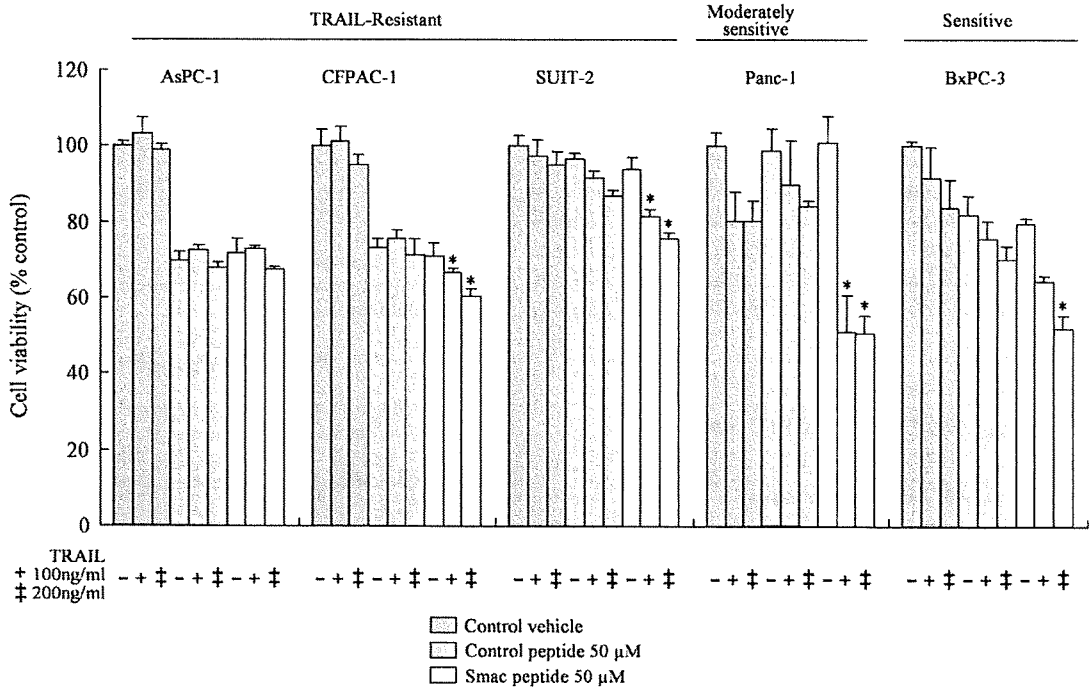


**FIG. 1.** Effect of Smac-peptide on TRAIL-induced apoptosis of pancreatic cancer cells. Cells were treated with various concentrations of TRAIL for 48 h in the presence of Smac peptide or the control peptide. Cell viability is shown as the mean percentage compared with untreated cells and bars represent the SD ($n = 3$). *, $P < 0.05$ for control peptide *versus* Smac peptide.

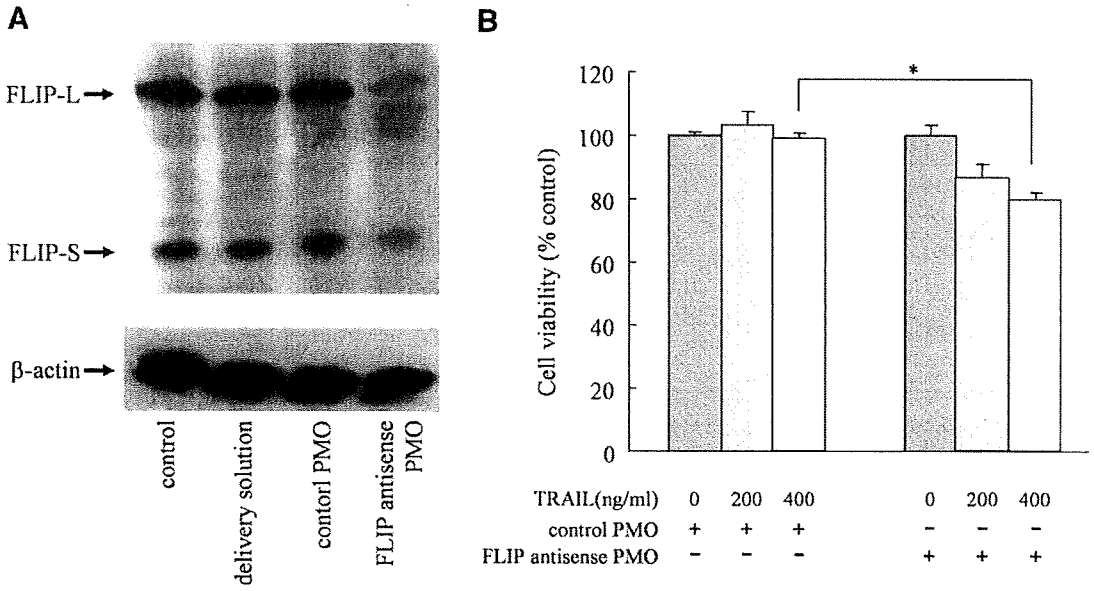


**FIG. 2.** Effect of FLIP antisense on FLIP expression and TRAIL sensitivity of TRAIL-resistant AsPC-1 cells. (A) Expression of FLIP-L and FLIP-S by immunoblot analysis with anti-FLIP monoclonal antibody. Expression of both FLIP-L and FLIP-S was decreased by FLIP antisense PMO. β-Actin was used to verify equal loading of proteins. (B) AsPC-1 cells were treated with TRAIL (0, 200, or 400 ng/mL) for 24 h after pretreatment with control PMO or FLIP antisense PMO. Cell viability is shown as the mean percentage compared with untreated cells and bars represent the SD ($n = 3$). *, $P < 0.05$ for control PMO *versus* FLIP antisense PMO.

peptide. TRAIL caused apoptosis of AsPC-1 cells in the presence of both FLIP antisense and Smac peptide, and the improvement of sensitivity to TRAIL was greater than the additive effect of FLIP antisense plus Smac peptide (Fig. 3A).

### Effect of Combined Use of FLIP Antisense and Embelin on TRAIL-Induced Apoptosis of AsPC-1 Cells

Because the combination of FLIP antisense and Smac peptide had an excellent effect, we next used the XIAP inhibitor embelin, which is derived from a natural benzoquinone product originally isolated from the Japanese herb Ardisia. We found that TRAIL effectively induced the death of AsPC-1 cells in the presence of FLIP antisense and embelin (Fig. 3B). Interestingly, embelin sensitized cells to TRAIL in a manner that was not dose-dependent.

### Effect of FLIP Antisense Plus Smac Peptide on Caspases and PARP

We next investigated the modulation of intracellular signaling in TRAIL-resistant AsPC-1 cells by the combination of FLIP antisense and Smac peptide. Exposure to TRAIL did not induce processing of caspases or cleavage of PARP, and the same results were obtained in the presence of Smac peptide. In the presence of FLIP antisense, however, partial cleavage of caspases-3 and -8 was observed. In the presence of both FLIP antisense and Smac peptide, exposure to TRAIL induced strong activation of caspases-3 and -8 as well as cleavage of PARP (Fig. 4).

## DISCUSSION

We have previously shown that TRAIL induces apoptosis to a variable extent in different pancreatic cancer cell lines [3]. Resistant cell lines (AsPC-1, CFPAC-1, and Suit-2) show strong expression of XIAP and FLIP, one of the splice variants of FLIP. Therefore, we investigated whether a Smac peptide containing the four N-terminal residues required for inactivation of XIAP or direct down-regulation of FLIP by using FLIP antisense could restore the sensitivity to apoptosis of TRAIL-resistant pancreatic cancer cells.

To address this question, we linked the six N-terminal residues of Smac protein to a cell membrane-penetrating polyarginine to facilitate intracellular delivery. Synthetic Smac N-terminal peptides fused to membrane-penetrating peptides have been found to bypass mitochondrial regulation and sensitize both cultured human cancer cells and tumor xenografts in mice [4–7]. In the presence of Smac peptide, TRAIL induced a significant and dose-dependent increase of the death of Suit-2, CFPAC-1, Panc-1, and BxPC-3 cells, but it showed no effect on TRAIL-resistant AsPC-1 cells which overexpress FLIP-S. Thus, AsPC-1 cells were still resistant to induction of apoptosis by TRAIL, even after inhibition of XIAP. These findings suggest that an additional block may be imposed by FLIP upstream of XIAP in