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Hepatitis B Virus Reactivation during Treatment with Multi-Tyrosine Kinase Inhibitor for Hepatocellular Carcinoma

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Key Words
Hepatitis B virus reactivation · Hepatocellular carcinoma · Multi-tyrosine kinase inhibitor

Abstract
Hepatitis B virus (HBV) reactivation is well documented in individuals with cancer who receive certain cytotoxic or immunosuppressive therapies including rituximab treatment. As a general rule, the risk is greatest upon withdrawal of chemotherapy. The risk ranges from approximately 20 to 50% among HBsAg-positive carriers. A 67-year-old man was diagnosed with inoperable multiple hepatocellular carcinoma accompanied by an increase in alpha-fetoprotein and protein induced by vitamin K absence or antagonist II level. Eighteen weeks after starting on the oral multi-tyrosine kinase inhibitor TSU-68, laboratory investigations showed a substantial increase in serum transaminase levels (AST: 302 IU/l; ALT: 324 IU/l) and an elevation of the HBV-DNA level (6.9 log copies/ml). The diagnosis was that the cause of the acute hepatitis was HBV reactivation and we immediately administered entecavir. Two months after the initiation of daily entecavir treatment, laboratory findings showed that the serum levels of transaminases and ALP had improved (AST: 18 IU/l; ALT: 10 IU/l; ALP: 197 U/l). When the HBV markers were examined 4 months later, they were altered: HBeAg was negative and HBeAb was positive. Entecavir treatment was discontinued after 6 months. Although reactivation with rituximab has been reported, reactivation with a tyrosine kinase inhibitor is extremely unusual in a patient who is HBsAg negative but anti-HBc positive. This is the first report describing HBV reactivation with an increasing HBV-DNA level in a HBsAg-negative/HBcAb-positive/HBsAb-positive patient who was treated with TSU-68 for hepatocellular carcinoma.

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Introduction

Hepatitis B virus (HBV) reactivation is well documented in individuals with cancer who receive certain cytotoxic or immunosuppressive therapies including rituximab treatment. As a general rule, the risk is greatest upon withdrawal of chemotherapy. The risk ranges from approximately 20 to 50% among hepatitis B surface antigen (HBsAg)-positive carriers. While any chemotherapy regimen can potentially lead to a reactivation of HBV replication, the risk may be decreased with steroid-free chemotherapy, implicating the use of glucocorticoids as a risk factor in lymphoma. In patients who are HBsAg negative but hepatitis B core antibody (HBcAb) positive, reactivation with rituximab has been reported.

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide and the third leading cause of cancer-related death [1]. Almost 80% of all cases are due to an underlying HBV and hepatitis C virus (HCV) infection. For advanced HCC patients, sorafenib, an inhibitor of vascular endothelial growth factor receptor-2 (VEGFR-2), c-Kit, and raf, has been demonstrated to be active and tolerable [2]. Scientific studies on the molecular pathogenesis of HCC have led to the active development of new drugs. TSU-68 is an orally administered, small-molecule, multiple receptor tyrosine kinase inhibitor that targets VEGFR-2, platelet-derived growth factor receptor, and fibroblast growth factor receptor [3]. Since it is a potent anti-angiogenic agent, TSU-68 is also expected to be effective against HCC.

This is the first report describing HBV reactivation in an HBsAg-negative/HBcAb-positive/hepatitis B surface antibody (HBsAb)-positive patient who was treated with the oral multi-tyrosine kinase inhibitor (multi-TKI) TSU-68 for HCC.

Case Report

A 67-year-old man was diagnosed with inoperable multiple HCC accompanied by an increase in alpha-fetoprotein (AFP) and protein induced by vitamin K absence or antagonist II level. Although the patient had neither undergone a blood transfusion nor been tattooed and although he did not drink alcohol or use illicit drugs, he had acquired acute hepatitis with no known cause at 30 years of age and had been treated for chronic hepatitis C with interferon-alpha at 58 years of age. When he was diagnosed with HCC, HBsAg, HCV antibody, and HCV-RNA were negative. HBsAb, HBcAb, and HBV-DNA levels were not assessed at the time of diagnosis. His family history of HBV included the following point of interest: his daughter was an HBV carrier. There was no information regarding his late wife's HBV status, as his wife had died almost a decade before.

Transcatheter arterial chemoembolization (TACE) was performed twice upon presentation. Once the patient showed reduction of tumor vascularity on angiography after TACE, he was registered for a phase II clinical trial with the new molecular agent TSU-68, which was administered as an adjuvant chemotherapy after TACE in accordance with the protocol used in the clinical trial. At the beginning of the adjuvant chemotherapy, serum transaminase levels were stabilized within the normal range (aspartate aminotransferase [AST]: 30 IU/l [normal: ≤33 IU/l]; alanine aminotransferase [ALT]: 32 IU/l [normal: ≤42 IU/l]). Eighteen weeks after starting on the novel treatment, laboratory investigations showed a substantial increase in serum transaminase levels (AST: 302 IU/l; ALT: 324 IU/l; fig. 1). The inhibitor was discontinued immediately. Computed tomography showed that the HCC was not exacerbated, and the serum AFP level was normal. Initiating a treatment with ammonium glycyrrhizate did not ameliorate the hepatocellular injury, and a gradual increase in transaminase levels was noted. The HBV markers were positive for HBsAg, HBcAb, and hepatitis B e antigen (HBeAg), and the quantity of HBV-DNA was 6.9 log copies/ml. HCV antibody and HCV-RNA were negative; the serum markers related to other hepatitis infections such as hepatitis A virus,

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cytomegalovirus, Epstein-Barr virus, and herpes virus were negative. The drug lymphocyte stimulation test yielded a negative result for TSU-68.

Using stored serums, we tested HBsAb, HBcAb, and HBV-DNA levels prior to the initiation of TSU-68; the sample was found to be HBsAb positive and HBcAb positive, with an HBV-DNA level of 2.1 log copies/ml. In addition, molecular analysis showed that the HBV genotype was C, with no HBV mutation in the pre-core or core promoter region. We therefore concluded that the cause of the acute hepatitis was HBV reactivation. We immediately administered entecavir at a dose of 0.5 mg once daily. Two months after starting the daily entecavir treatment, laboratory findings showed that the serum levels of transaminases and alkaline phosphatase (ALP) had improved (AST: 18 IU/l; ALT: 10 IU/l; ALP: 197 U/l). When the HBV markers were examined 4 months later, they were altered: HBeAg was negative and hepatitis B e antibody was positive. Entecavir treatment was discontinued after 6 months, but we continued to observe the patient and carefully monitor his liver function and HCC. One year after commencing the treatment for HBV reactivation, HBsAg was negative, HBsAb was positive, and the quantity of HBV-DNA was undetectable by real-time polymerase chain reaction. After the withdrawal of entecavir, no evidence of increased liver damage or disease progression has been noted during follow-up up to today.

Discussion

To the best of our knowledge, this is the first report describing HBV reactivation in an HBsAg-negative/HBcAb-positive/HBsAb-positive patient who was treated with the multi-TKI TSU-68 for HCC in a clinical trial.

Hepatic flare causes an elevation in serum transaminase levels, with the proposed definition of hepatic flare constituting an abrupt increase in serum ALT level to 3–5 times higher than the normal range [4]. HBV flares are usually preceded by an increase in serum HBV-DNA levels. However, because the increase in serum ALT level lags behind the increase in HBV-DNA level, serum HBV-DNA levels may be declining or undetectable when patients with flares are initially evaluated. Thus, HBV reactivation is closely related to the increased quantity of HBV-DNA. In the present patient, who was HBsAg negative, HBsAb positive, and HBcAb positive, serum ALT level increased to more than 7 times the upper limit of the normal range, suggesting a hepatitis flare; the simultaneous increase in the quantity of HBV-DNA allowed us to diagnose HBV reactivation.

HBV reactivation is known to occur often in individuals with chronic HBV infection. In patients with cancer who are HBsAg positive, especially in those with leukemia and lymphoma, the administration of corticosteroids and rituximab during a hematopoietic cell transplantation therapy has been noted to influence HBV reactivation [5]. Recently, it has been reported that the use of rituximab results in HBV reactivation in individuals who are HBsAg negative/HBcAb positive or HBsAb positive. This finding suggests that HBsAg-positive patients, as well as HBsAg-negative/HBcAb-positive or HBsAb-positive patients, have a high risk of HBV reactivation [6]. The guidelines issued by the American Association for the Study of Liver Diseases and the American Society of Clinical Oncology Provisional Clinical Opinion recommend that persons receiving cytotoxic or immunosuppressive therapy should be tested for serologic markers of HBV infection so that prophylactic antiviral therapy can be administered to prevent reactivation in HBsAg-positive patients [7].

Apart from rituximab, other drugs that induce HBV reactivation include infliximab, which targets tumor necrosis factor and is used for the treatment of rheumatoid

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arthritis and inflammatory bowel disease [8], and alemtuzumab, which is a humanized monoclonal antibody directed against CD52 that is used for the treatment of chronic lymphocytic leukemia [9]. However, few reports have described small-molecule inhibitors, such as the histone deacetylase inhibitor [10] that is used for T-cell lymphoma and imatinib, which is used for chronic myeloid leukemia. We believe that this report is unique in that it describes HBV reactivation during the administration of a novel multi-TKI in a patient who did not have any hematopoietic disease and who was HBsAg negative, HBsAb positive, and HBcAb positive. In the SHARP trial for advanced HCC, the multi-TKI sorafenib led to liver dysfunction, which resulted in 5% of the patients discontinuing treatment, but no reports have described an association between such liver dysfunction and HBV reactivation [11]. Although liver dysfunction and an elevation of AST and ALT was noted in 29% of the patients with HCC in a phase II study of TSU-68 which was also administered to our patient [12], our case highlights the possibility that HBV reactivation may be latent in such patients.

HBV genotype influences clinical outcomes, serum quantitative HBV-DNA levels, fulminant hepatitis, and mutational patterns in the pre-core and core promoter regions [13]. In the patient we presented, the HBV genotype was C, but the serum HBV-DNA level at the time of initiating treatment with TSU-68 was not high, and no HBV mutations were observed in the pre-core or core promoter region. These findings lead us to surmise that HBV genotype directly influences HBV reactivation associated with chemotherapy. On the other hand, it should also be noted that no depletion of neutrophils or lymphocytes occurred in our patient. The inhibition of tyrosine kinase may be relevant to the replication of HBV, although it remains unclear how tyrosine kinase inhibition induces HBV reactivation.

Preemptive therapy with lamivudine for HBsAg-positive patients undergoing chemotherapy reduces the risk of HBV reactivation and HBV-associated morbidity and mortality [14]. Monotherapy with entecavir in adult patients with chronic HBV infection is safe, tolerable, and lowers serum HBV-DNA levels to a greater extent than lamivudine [15]. A clinical trial assessing entecavir as preventive therapy for HBV reactivation associated with rituximab treatment is currently underway. It is unclear whether preventive antiviral therapy for patients receiving multi-TKIs is effective, and the risk of developing HBV reactivation during such treatment warrants further investigation.

In conclusion, the risk of HBV reactivation associated with multi-TKIs, especially those that inhibit angiogenesis and cell growth, remains unclear. In clinical trials of such new agents, it is difficult to predict the time point at which HBV reactivation occurs following the administration of new molecular agents. Risk classification of chronic HBV infection and preemptive therapy may prevent HBV reactivation and contribute to the development of novel anticancer treatments in this patient population.

Disclosure Statement

The authors declare that they have no potential conflicts of interest to disclose.

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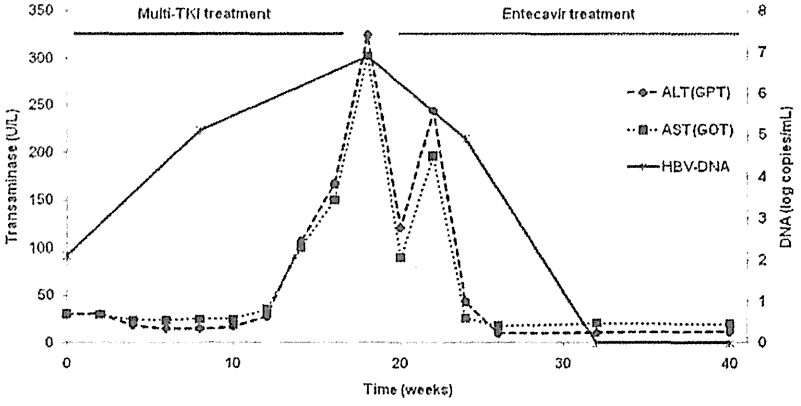

Fig. 1. Changes in serum transaminase levels and HBV-DNA level.

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Altered Plasma Apolipoprotein Modifications in Patients with Pancreatic Cancer: Protein Characterization and Multi-Institutional Validation

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Abstract

Background: Among the more common human malignancies, invasive ductal carcinoma of the pancreas has the worst prognosis. The poor outcome seems to be attributable to difficulty in early detection.

Methods: We compared the plasma protein profiles of 112 pancreatic cancer patients with those of 103 sex- and age-matched healthy controls (Cohort 1) using a newly developed matrix-assisted laser desorption/ionization (oMALDI) QqTOF (quadrupole time-of-flight) mass spectrometry (MS) system.

Results: We found that hemi-truncated apolipoprotein AII dimer (ApoAII-2; 17252 *m/z*), unglycosylated apolipoprotein CIII (ApoCIII-0; 8766 *m/z*), and their summed value were significantly decreased in the pancreatic cancer patients [$P = 1.36 \times 10^{-21}$, $P = 4.35 \times 10^{-14}$, and $P = 1.83 \times 10^{-24}$ (Mann-Whitney *U*-test); area-under-curve values of 0.877, 0.798, and 0.903, respectively]. The significance was further validated in a total of 1099 plasma/serum samples, consisting of 2 retrospective cohorts [Cohort 2 ($n = 103$) and Cohort 3 ($n = 163$)] and a prospective cohort [Cohort 4 ($n = 833$)] collected from 8 medical institutions in Japan and Germany.

Conclusions: We have constructed a robust quantitative MS profiling system and used it to validate alterations of modified apolipoproteins in multiple cohorts of patients with pancreatic cancer.

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Competing Interests: HK and TS are the employees of Mitsui Knowledge Industry, and YO and NO used to belong to Molecuence Corporation. This does not alter the authors' adherence to all the PLoS ONE policies on sharing data and materials. The authors have declared that no other competing interests exist.

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Introduction

With a 5-year survival rate of less than 10%, invasive ductal carcinoma of the pancreas has the worst prognosis among the more common human malignancies [1,2,3]. The poor outcome of pancreatic cancer patients seems to be attributable to difficulty in early detection. Most patients with early-stage pancreatic cancer have no symptoms, and do not visit clinics until the disease has progressed to an advanced stage. Because surgical resection is currently the only curative treatment for pancreatic cancer, early detection before the development of metastasis is essential to improve its outcome. However, no screening method has been established for pancreatic cancer [2]. Enhanced computed tomography (CT) and positron emission tomography (PET) are useful for the diagnosis of pancreatic diseases, but these modalities are potentially hazardous and would probably be too labor-intensive and cost-ineffective for mass screening, because of the relatively low incidence of pancreatic cancer.

The circulating blood proteome holds great promise as a reservoir of information that could be used for the diagnosis of various physiological and pathological conditions [4]. We

previously found a plasma biomarker set that was able to distinguish pancreatic cancer patients including those with stage I and II disease from healthy individuals by mass spectrometry (MS)-based proteomics [5]. However, it was not possible to identify the marker proteins, and the numbers/sources of cases and types of disease were limited [6].

In the present study, we determined the amino acid sequences and modifications of marker proteins and validated their significance in larger cohorts. Here we report that the two modified forms of plasma/serum apolipoproteins are reduced in patients with pancreatic diseases.

Materials and Methods

Patient Samples

Four cohorts (namely Cohorts 1–4) consisting of a total of 1314 plasma or serum samples were collected at the following medical institutions in two countries (Japan and Germany) (Tables S1 and S2):

Cohort 1. 215 plasma samples [sex- and age-matched patients with histologically or cytologically proven pancreatic ductal adenocarcinoma ($n = 103$) and healthy controls ($n = 112$)] collected at the National Cancer Center Hospital (NCCH) (Tokyo, Japan) and Tokyo Medical University Hospital (TMUH) (Tokyo, Japan) between August 2002 and February 2005, as reported previously [5].

Cohort 2. 103 plasma samples [sex- and age-matched pancreatic cancer patients ($n = 62$) and healthy controls ($n = 41$)] newly collected at the NCCH between August 2003 and May 2005.

Cohort 3. 163 serum samples [pancreatic cancer patients ($n = 52$), patients with chronic pancreatitis ($n = 58$) and healthy controls ($n = 53$)] collected at the Department of General Surgery, University of Heidelberg (Heidelberg, Germany) between 2003 and 2006 [7,8].

Cohort 4. 833 plasma (and serum) samples collected prospectively from 7 medical institutions in Japan [NCCH, Osaka National Hospital (Osaka, Japan), Jichi Medical School Hospital (Shimotsuke, Japan), Osaka Medical Center for Cancer and Cardiovascular Diseases (Osaka, Japan), TMUH, Osaka Medical College Hospital (Osaka, Japan), and Fukuoka University Hospital (Fukuoka, Japan)] between August 2006 and October 2008 for this study. A document detailing standard operating procedures was distributed to each institute, and all the plasma samples were collected prospectively under identical conditions. This was an essentially hospital-based cohort consisting of healthy volunteers and newcomers to mainly gastrointestinal services at the participating hospitals. The final diagnoses of the patients were collected separately from their blood samples. Written informed consent was obtained from every subject. The study protocol was reviewed and approved by the ethics committee of each participating institution.

The collection and storage conditions (such as collection tubes, anticoagulants, temperature, and number of thawing and freezing cycles) were kept strictly identical among plasma samples within the same cohort. Apparently hemolyzed samples were excluded. All the samples were blinded and randomized prior to MS analysis (detailed procedures are available in Methods S1). Only plasma samples were used for the MS analysis of Cohort 4. To ensure reproducibility of measurements, the samples in Cohort 4 were analyzed independently by two investigating teams at different laboratories [NCCRI (Tokyo, Japan) and Molecuence Corporation (Yokohama, Japan)].

Immunoprecipitation and Immunoblotting

Anti-pan ApoAII rabbit polyclonal antibody was purchased from Abcam (Cambridge, UK). Immunoprecipitation was performed using immunoaffinity chromatography (IAC) Protein G beads (Bruker Daltonics). Two rabbit polyclonal antibodies were raised against ApoAII monomer peptides with -ATQ and -AT C-termini. cDNA fragments of ApoAII (encoding amino acids 1–100 and 1–99; NP_001634) were amplified by PCR and subcloned into pGEX-6P-2 plasmids (GE Healthcare). N-terminally glutathione S-transferase (GST)-tagged fusion proteins were expressed in *Escherichia coli* and affinity purified on glutathione-Sepharose 4B (GE Healthcare) as described previously [9]. The reactivity of antibodies with the GST-tagged proteins was assessed as described previously [10].

Protein samples were separated by SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). Blots were visualized with an enhanced chemiluminescence kit (GE Healthcare) and quantified as described previously [11].

CA19-9 Measurement

Serum samples in Cohort 4 were analyzed using commercially available chemiluminescence enzyme immunoassay kits for CA19-9 (LUMIPULSE Presto CA19-9, Fujirebio Inc., Tokyo, Japan) [5].

Statistics

Statistically significant differences were detected using Mann-Whitney *U*-, Student *t*-, and χ^2 tests. Receiver operator characteristic (ROC) curves were generated and AUC values were calculated using StatFlexs software (version 5.0; Artech, Osaka, Japan) and the modules of R-project (http://www.r-project.org/) [5,12,13].

Results

Development of MS Measurement with High Reproducibility

We developed a plasma protein profiling system by employing automated hydrophobic chromatography and oMALDI QqTOF-MS. In a quality control experiment, 2173 independent low-molecular-weight (within the range 1000–30,000 m/z) protein peaks were detected from a 5-μl sample of mixed plasma from healthy volunteers. The average correlation coefficient (CC) value for all 2173 peaks among triplicate measurements was 0.983 (Fig. 1A). The reproducibility of measurement was further confirmed by calculating the coefficient of variation (CV) values for peaks of interest (see below) (Fig. 1B).

We prepared a large stock of plasma mixture as a quality control standard, and two aliquots of the stock were processed simultaneously with every 22 test samples. The CC value of the standard was monitored to ensure consistency of measurement for every plate (Fig. 1C–E).

Protein Identification by Tandem Mass Spectrometry (MS/MS)

Quantitative plasma MS data were obtained from 103 patients with pancreatic cancer and 112 sex- and age-matched healthy controls (Cohort 1). We found a 17252-m/z MS peak (Fig. 2, indicated by double asterisks) that stood out as having the highest statistical significance ($P = 1.36 \times 10^{-21}$, Mann-Whitney *U*-test) (Fig. S1). The molecular weight was almost identical to that of a marker protein we had identified previously using a different MS system [5]. MS/MS analysis and database/literature searches

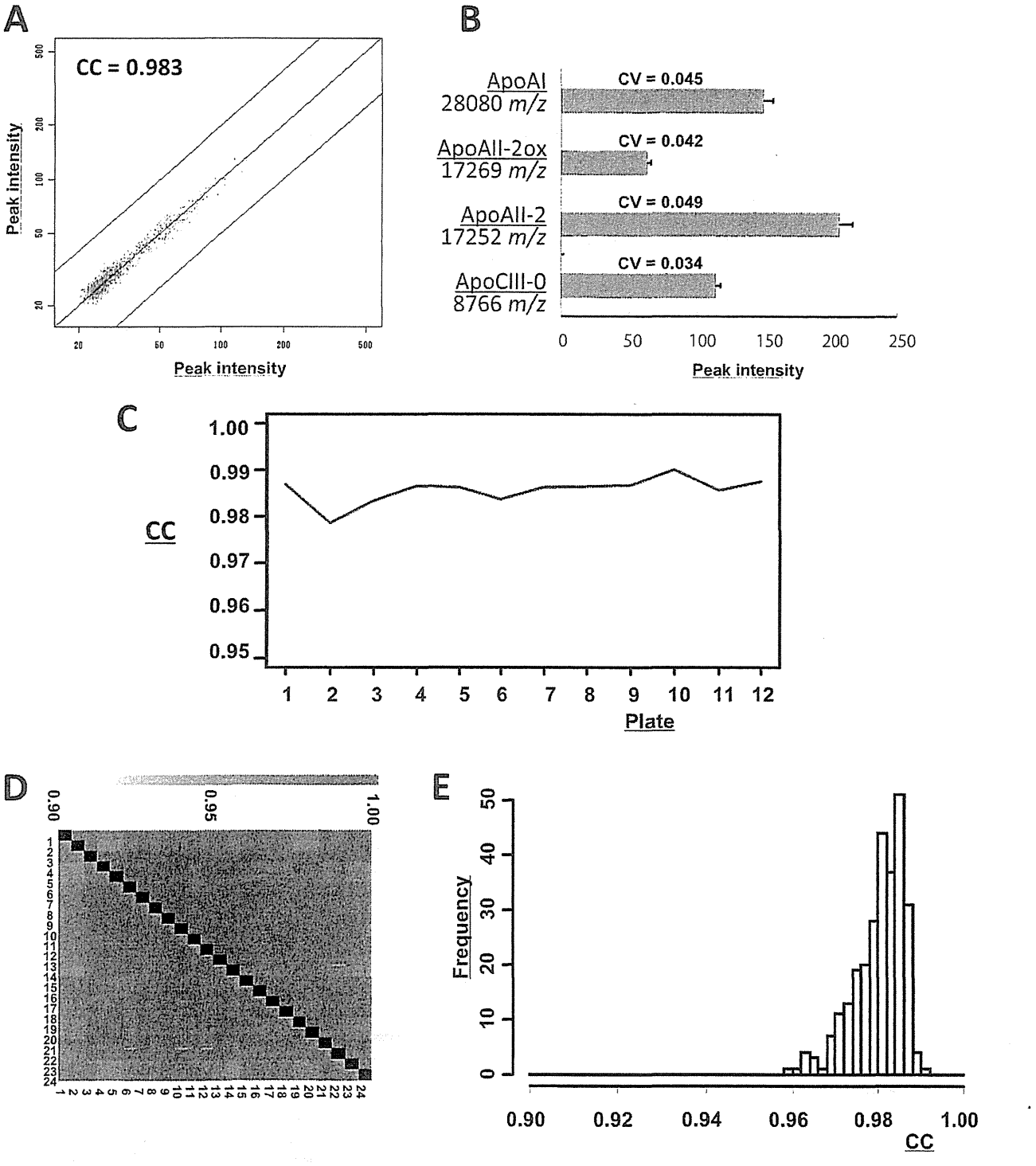

Figure 1. Reproducibility of automated chromatography and oMALDI-QqTOF-MS. (**A**) Two-dimensional plot analysis showing the correlation of 2173 corresponding peaks between two of the triplicate measurements (indicated in red, blue, and green) of a standard plasma mixture. Lines indicate 2-fold differences. (**B**) A standard plasma mixture was analyzed 24 times, and the CV values [= SD (bars)/mean (columns)] of representative protein peaks (ApoAI, ApoAII-2ox, ApoAII-2, and ApoCIII-0) were calculated. (**C**) Transition of CC values between the duplicate measurements of a standard plasma mixture in the 12 plates (24 measurements) of Cohort 1. (**D and E**) Correlation matrix (**D**) and distribution (**E**) of mutual similarity (CC values) for the 24 duplicate measurements (1–24).18.
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revealed that the 17252-m/z protein was a heterodimer of ApoAII, one peptide chain of which lacked a glutamine (Q) residue at the C-terminus (-ATQ/−AT) (namely, ApoAII-2) (Figs. S2 and S3) [7,14]. The oxidized form of ApoAII-2 (17269 m/z, ApoAII-2ox) [14] showed the second highest statistical significance ($P = 5.14 \times 10^{-20}$) (Table S3).

In order to confirm the identity of the protein, we carried out immunoprecipitation with an antibody specific to ApoAII, and the immunoprecipitated proteins were analyzed by oMALDI-QqTOF-MS (Fig. S4). ApoAII-2 (-ATQ/−AT) and its oxidized form (ApoAII-2ox) were detected along with other post-translationally modified forms of ApoAII [AII-1 (17380 m/z) and AII-3 (17124 m/z)]. ApoAII-1 is a homodimer of untruncated ApoAII peptides with C-terminal ends of -ATQ/−ATQ. ApoAII-3 is a homodimer of AII peptide chains, both the C-terminal ends of which lack glutamine (Q) residues (-AT/−AT) (Fig. 3) [14].

Another protein with a molecular size of 8766 m/z also showed a highly significant difference ($P = 4.35 \times 10^{-14}$) between pancreatic cancer patients and healthy controls (Fig. 2, indicated by quadruple asterisks). The molecular size of the protein was also almost identical to that of one of 4 marker proteins that we had reported in our previous study [5]. MS/MS analysis (Fig. S5) identified the 8766-m/z protein as ApoCIII, which is a 79-amino-acid glycoprotein known to have three differently glycosylated isoforms {unglycosylated (CIII-0), monosialyated (CIII-2), and disialylated (CIII-2) [15,16]}, and the 8766-m/z protein corresponds to ApoCIII-0. The identity was confirmed by MS analysis of proteins immunoprecipitated with anti-ApoCIII antibody (data not shown).

Combination of ApoAII-2 and ApoCIII-0 and its Validation

ApoAII-2 and ApoCIII-0 were able to differentiate the 103 pancreatic cancer patients from the 112 healthy controls (Cohort

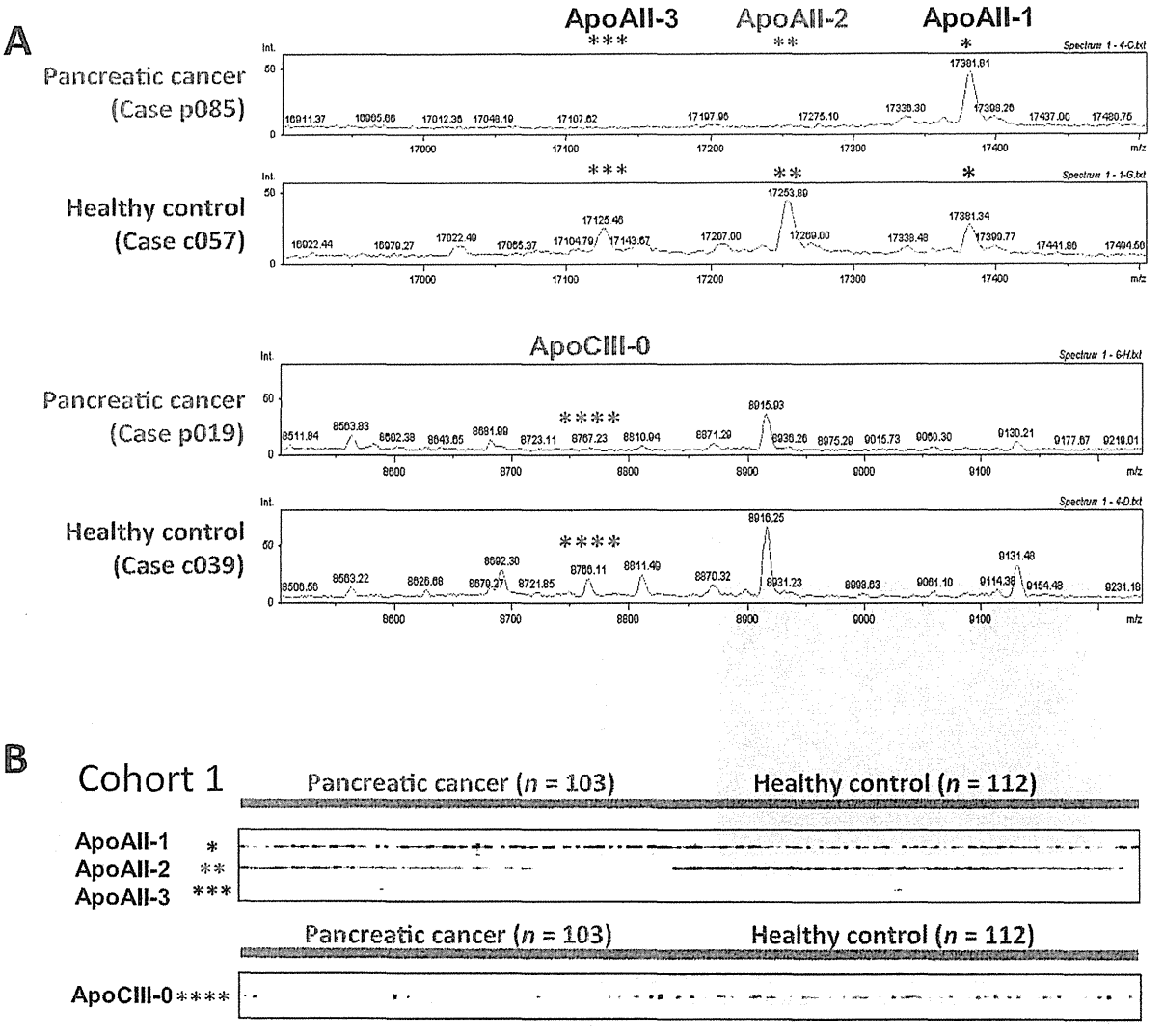

Figure 2. oMALDI-QqTOF-MS profiling of plasma proteins. (**A**) Spectra for representative pancreatic cancer patients and controls within the ranges 16,900 to 17,500 m/z (top) and 8500 to 9250 m/z (bottom). (**B**) Gel-like images of ApoAII and ApoCIII-0 in Cohort 1 (215 cases). (**A** and **B**) Black single asterisks (*), red double asterisks (**), black triple asterisks (***), and red quadruple asterisks (****) indicate MS peaks of ApoAII-1, ApoAII-2, ApoAII-3, and ApoCIII-0, respectively.
doi:10.1371/journal.pone.0046908.g002

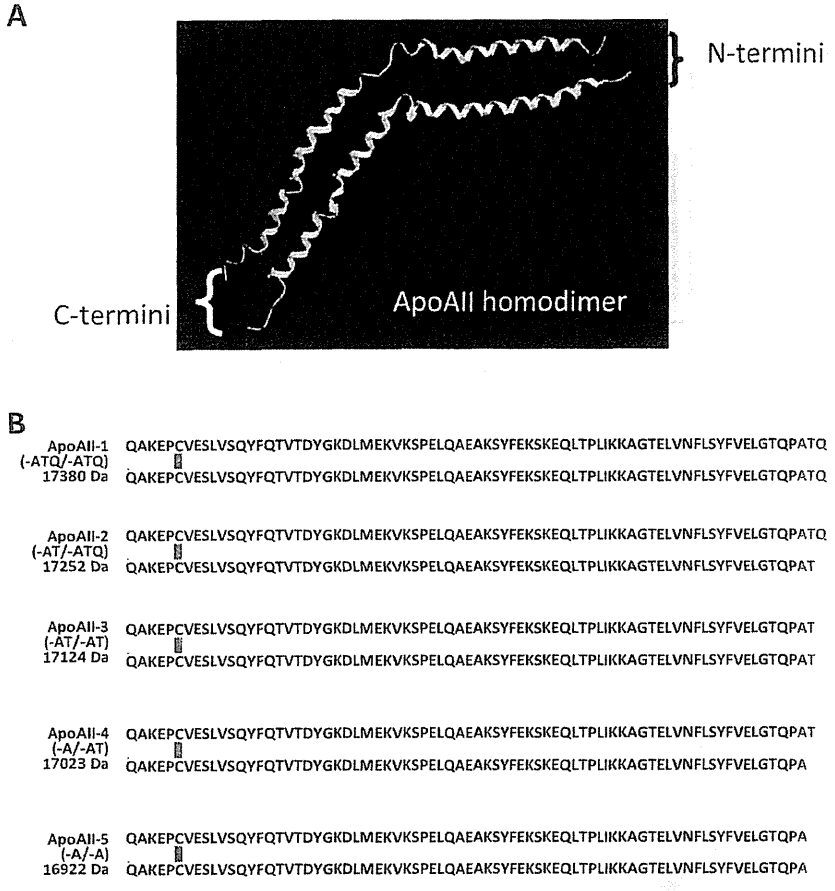

Figure 3. Structure of ApoAII dimers. (**A**) Predicted three-dimensional structure of dimerized ApoAII protein. The model was built using the MOE software package (Ryoka Systems Inc., Tokyo, Japan). Red color indicates a disulfide bond. (**B**) Amino acid sequences and calculated theoretical molecular masses of ApoAII homo/heterodimers (ApoAII-1 to -5). Two peptides were dimerized via an N-terminal disulfide bond.
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1) with an area-under-curve (AUC) value of 0.877 and 0.798, respectively (Fig. 4A and Table S3). Because both ApoAII-2 and ApoCIII-0 were decreased in pancreatic cancer patients and the distribution was mutually independent (Fig. S6), simple summing of the two biomarkers improved the AUC value to 0.903.

The high discrimination capability of ApoAII-2, ApoCIII-0, and their summed value (hereafter denoted as ApoAII-2+CIII-0) between pancreatic cancer patients and controls was validated in an independent cohort consisting of 41 sex- and age-matched healthy controls and 62 pancreatic cancer patients (Cohort 2) (Fig. 4B and Table S3).

To confirm the universality of this discrimination across different ethnicities, we next examined 163 serum samples collected in Germany (Cohort 3) (Fig. 4C and Table S4) [7,8]. The reduction of ApoAII-2 and ApoCIII-0 in pancreatic cancer patients was evident even in this cohort. However, the decrease of ApoAII-2 and ApoCIII-0 was found not to be specific to pancreatic cancer. ApoAII-2+CIII-0 was decreased significantly in patients with chronic pancreatitis ($P = 8.86 \times 10^{-11}$).

The mean age of healthy controls in Cohort 3 (39.1 years) was significantly lower that that of patients with pancreatic cancer (63.1 years) or pancreatitis (50.3 years) (Table S1). Therefore, the possibility that the extremely high value of ApoAII-2 (0.958) in this cohort was attributable to this age difference cannot be excluded.

Prospective Multi-institutional Validation

Plasma/serum protein profiling by direct MS was introduced as a revolutionary new tool for biomarker discovery [17,18]. However, its validity has been frequently discussed [6,19], mainly on the basis of possible biases in sample collection, storage, and freeze/thawing, as well as poor mass accuracy, thus creating difficulty with protein identification. To e6nsure the absence of any sampling bias, we then collected plasma samples prospectively from 7 medical institutions in Japan (Cohort 4) using the same predetermined protocol of blood collection, storage, and freeze/thawing. This multi-institutional collaborative study group was organized by the "Third-Term Comprehensive Control Research for Cancer" conducted by the MHLWJ and affiliated to the International Cancer Biomarker Consortium (ICBC) (http://www.fhcrc.org/science/international_biomarker/) and follows the principle of PRoBE design (prospective specimen collection before outcome ascertainment and retrospective blinded evaluation).

All the prospectively collected samples were blinded, randomized, and sent to a laboratory at the Molecuence Corporation. Even this independent/blinded validation showed that pancreatic

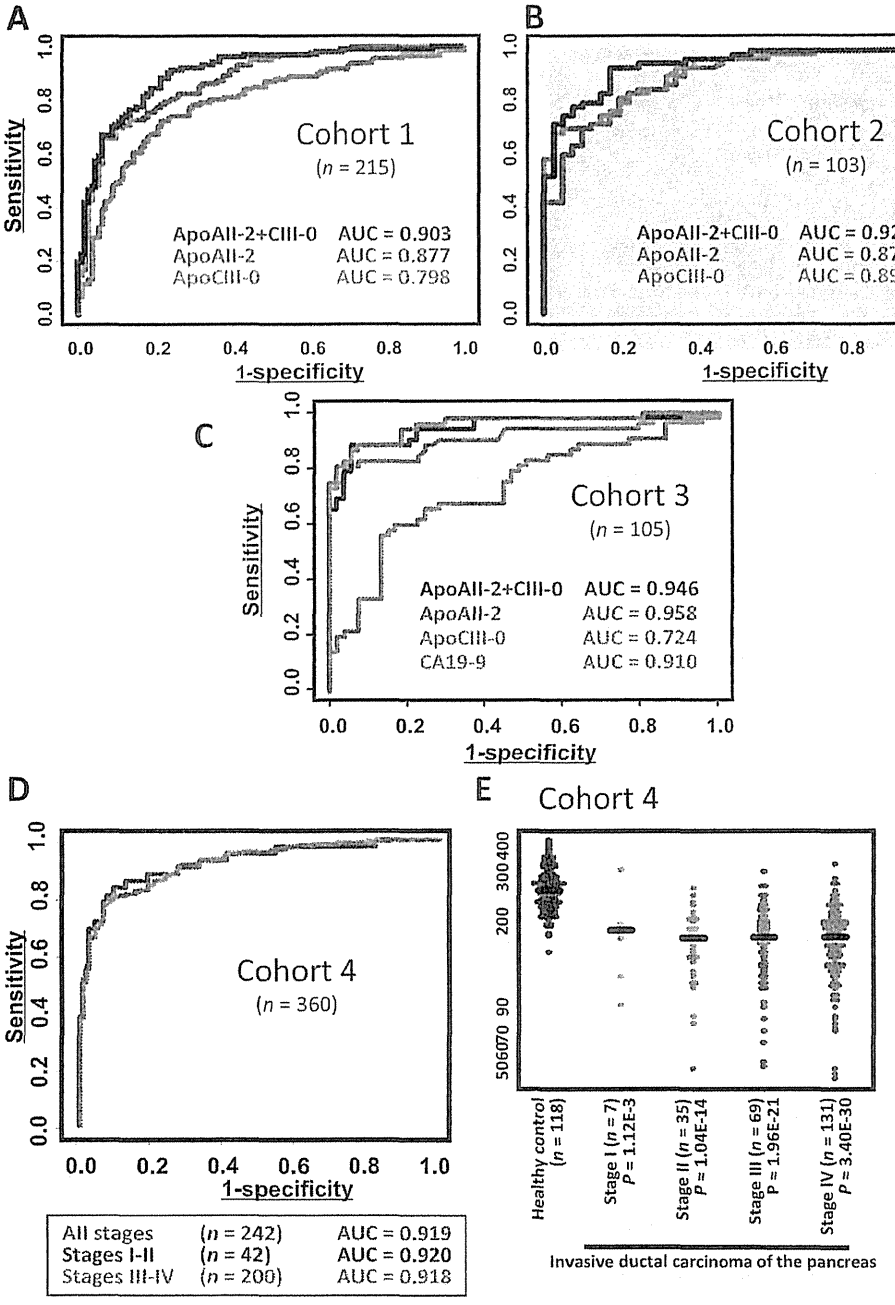

Figure 4. Reduction of ApoAII-2 and ApoCIII-0 in pancreatic cancer. (**A and B**) ROC analysis of ApoAII-2, ApoCIII-0, and their combination (ApoAII-2+CIII-0) in Cohorts 1 (A) and 2 (B). (**C**) ROC analysis showing the capacity of ApoAII-2, ApoCIII-0, ApoAII-2+CIII-0, and CA19-9 for discrimination of pancreatic cancer patients from healthy controls in the German cohort (Cohort 3). (**D**) ROC analysis of ApoAII-2+CIII-0 in Cohort 4 according to clinical stage (Cohort 4). (**E**) Distribution of ApoAII-2+CIII-0 according to clinical stage. Lines indicate median values (Cohort 4).
doi:10.1371/journal.pone.0046908.g004

cancer patients, including those with early-stage disease, were readily distinguishable from healthy controls using the combination of ApoAII-2 and ApoCIII-0 (ApoAII-2+CIII-0). The AUC values for ApoAII-2+CIII-0 were 0.920, 0.918, and 0.919 for pancreatic cancer patients at clinical stages I-II, III-IV, and all stages [TNM classification, UICC (International Union against Cancer)], respectively (Fig. 4D).

Comparison and Combination with CA19-9

The combination of ApoAII-2 and ApoCIII-0 (ApoAII-2+CIII-0) was able to detect 66.1% (160/242) of pancreatic cancer patients relative to healthy individuals (Table S5) at a cut-off value [189 arbitrary units (AU)] that had been arbitrarily determined to obtain a specificity of 95% or higher [97.5% (115/118)]. With this cut-off, the sensitivity of ApoAII-2+CIII-0 became inferior to that

of CA19-9 [79.8% (193/242)], but the specificity was superior to that of CA19-9 [94.9% (112/118)], and the AUC value of ApoAII-2+CIII-0 was almost equal to that of CA19-9. However, the reduction of ApoAII-2+CIII-0 was not influenced by the clinical stage of the disease, and was detectable even in patients with stage I pancreatic cancer (Fig. 4E). The AUC value of ApoAII-2+ApoCIII0 for stage I pancreatic cancer (0.868) was higher than that of CA19-9 (0.774) (Table S5), suggesting the potential advantage of ApoAII-2+ApoCIII0 over CA19-9 for detection of early-stage pancreatic cancer. However, the number of stage I pancreatic cancer patients examined in this study was still small (7 cases), and this issue remains to be confirmed.

It was noteworthy that 93.4% (226/242) of patients with pancreatic cancer were detected either by ApoAII-2+CIII-0 or by CA19-9 without compromising the specificity for healthy individuals [93.2% (110/118)]. Furthermore, ApoAII-2+CIII-0 was able to detect a broad spectrum of diseases in the pancreatic and hepatobiliary region with a sensitivity of >60% (Table 1). However, the reduction of ApoAII-2+CIII-0 did not seem to be specific to pancreatic and hepatobiliary diseases. Patients with esophageal, gastric, and colorectal cancers also showed reduction of plasma ApoAII-2+CIII-0 with a frequency of 36.4% (4/11), 23.9% (34/142), and 32.4% (46/142), respectively.

Similar significance was reproducibly obtained by MS measurements conducted independently at the National Cancer Center Research Institute (NCCRI) (Fig. 5 and data not shown).

Novel Modification of ApoAII

The majority (>95%) of circulating ApoAII protein molecules form N-terminally disulfide-linked dimers. In addition to the 3 known isoforms of ApoAII (ApoAII-1 to -3) [14] we newly identified two other differently modified ApoAII dimers (ApoAII-4 and AII-5) (Figs. 3 and 5):

ApoAII-4 (17023 *m/z*), which lacks C-terminal glutamine (Q) and threonine (T) residues from one peptide and a glutamine residue from the other (-A/−AT).

ApoAII-5 (16922 *m/z*), which lacks C-terminal glutamine and threonine residues from both peptides (-A/−A).

ApoAII-1 (intact ApoAII with -ATQ/−ATQ termini) was not decreased in pancreatic cancer patients (Fig. 5A), indicating that the decrease of ApoAII-2 is not caused by a simple reduction of overall protein production, but by protein modification. The specific increase of ApoAII-5 in pancreatic cancer patients indicates that accelerated digestion may be one of the mechanisms responsible for the reduction of ApoAII-2 (see the Discussion section).

Production of antibody specific to ApoAII peptide with a -ATQ or -AT end. To verify the truncation of ApoAII in patients with pancreatic diseases, we newly produced a polyclonal antibody that specifically recognizes the ApoAII peptide with an intact C-terminus (-ATQ), but not that with a cleaved C-terminus (-AT) (Fig. 6A), as well as a polyclonal antibody that specifically recognizes the Apo-AII peptide with a cleaved (-AT) C-terminus, but not that with an intact (-ATQ) C-terminus (Fig. 6B).

The results of immunoblot analyses using these antibodies (Fig. 6C) were consistent with those obtained by oMALDI-QqTOF-MS (Fig. 6D). In 2 pancreatic cancer patients (lanes g and h) who lacked ApoAII-1 and ApoAII-2 but possessed ApoAII-4 and ApoAII-5 (Fig. 6D), immunoblot analysis under non-denaturing (native) conditions revealed loss of the 17-kDa ApoAII peptide detectable with anti-ApoAII (-ATQ) antibody (middle, Fig. 6C), and a faster-migrating (smaller) ApoAII peptide was detected with anti-Pan Apo-AII antibody (top, indicated by asterisks, Fig. 6C).

Discussion

Pancreatic cancer is a devastating disease, but a substantial proportion of patients can be cured if detection is possible at an

Table 1. ApoAII-2+ApoCIII-0 positivity rates in various gastrointestinal diseases (Cohort 4).

	n	ApoAII-2+ApoCIII-0 (<189.0 AU)	CA19-9 (>37.0 U/ml)	ApoAII-2+ApoCIII-0 or CA19-9
Healthy control	118	2.54% (3/118)	5.08% (6/118)	6.78% (8/118)
Invasive ductal adenocarcinoma of the pancreas	243[a]	**65.8% (160/243)**	**79.8% (194/243)**	**93.4% (227/243)**
Other malignant tumor of the pancreas	18	**72.2% (13/18)**	22.2% (4/18)	**83.3% (15/18)**
Benign tumor or cyst of the pancreas	37	35.1% (13/37)	8.18% (3/37)	37.8% (14/37)
Chronic pancreatitis	14	**78.6% (11/14)**	14.3% (2/14)	**85.7% (12/14)**
Hepatocellular carcinoma	13	**69.2% (9/13)**	15.4% (2/13)	**69.2% (9/13)**
Carcinoma of the duodenum	10	**70.0%(7/10)**	30.0% (3/10)	**90.0% (9/10)**
Gallbladder or cholangiocellular carcinoma	44	56.8% (25/44)	**63.6% (28/44)**	**81.8% (36/44)**
Benign disease of gallbladder or bile duct	21	28.6% (6/21)	14.3% (3/21)	33.3% (7/21)
Esophageal cancer	11	36.4% (4/11)	18.2% (2/11)	36.4% (4/11)
Gastric cancer	142	23.9% (34/142)	16.2% (23/142)	33.1% (47/142)
Colorectal cancer	142	32.4% (46/142)	17.6% (25/142)	40.8% (58/142)
Gastrointestinal stromal tumor	3	0% (0/3)	0%(0/3)	0% (0/3)

[a]Includes one case of unknown clinical stage. Positivity rates higher than 60% are highlighted in boldface.
doi:10.1371/journal.pone.0046908.t001

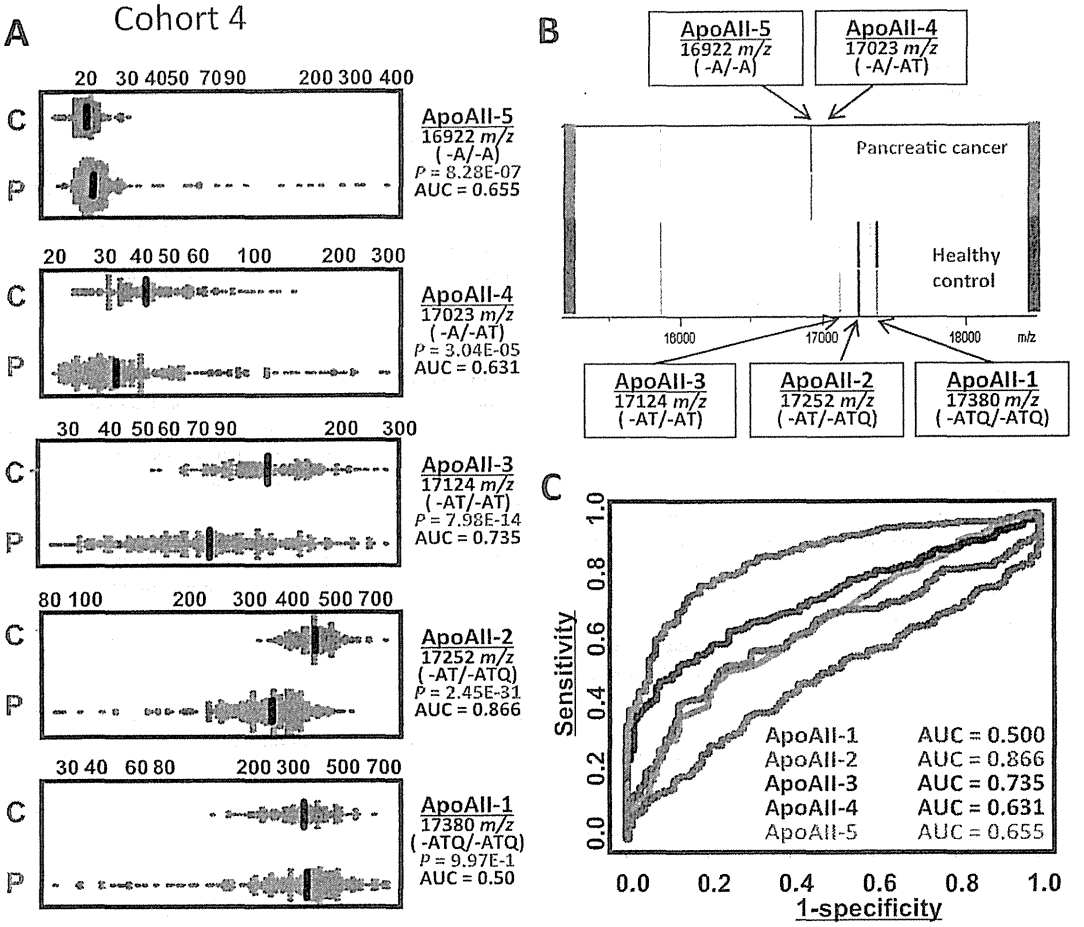

Figure 5. Differential modification of ApoAII. Distribution (peak intensity in arbitrary units) (**A**), spectra of representative cases (**B**), and ROC analysis (**C**) of the indicated modified forms of ApoAII in Cohort 4. C, healthy control ($n = 128$); P, pancreatic cancer ($n = 249$).
doi:10.1371/journal.pone.0046908.g005

early stage [20,21]. To improve the outcome of pancreatic cancer, establishment of an effective screening method is essential. Although the decrease of the modified plasma/serum apolipoproteins reported here is not specific to pancreatic cancer, this test is minimally invasive and in this sense might be considered for application to primary screening of asymptomatic patients with pancreatic cancer as well as other gastrointestinal diseases among the general (apparently healthy) population, if followed by an appropriate second screening for differential diagnosis, such as enhanced CT or ultrasonography [22]. Screening of blood by MS would provide asymptomatic patients with a precious chance to receive clinical attention and eventually increase the rate of discovery of early-stage pancreatic cancer. However, the sensitivity and specificity of the current set (ApoAII-2+CIII-0), even when combined with CA19-9, does not seem to be sufficient, and the number of patients with early-stage pancreatic cancer examined in this study was small. It will be necessary to conduct a prospective randomized trial before definite conclusions can be drawn about the utility of reduced ApoAII-2 and ApoCIII-0 in the setting of cancer screening.

Most tumor markers secreted by cancer cells into the circulation do not show a significant increase when cancer is in its early stage and the tumor burden relative to the entire body is small. This nature of most conventional tumor markers makes their application to cancer screening difficult [3,23]. However, unlike conventional tumor markers, the modified apolipoproteins were reduced in patients with pancreatic cancer and other gastrointestinal diseases. Apolipoproteins are produced mainly in the liver, and ApoAII and ApoCIII proteins are unlikely to be produced by cancer cells. However, 17252-m/z ApoAII heterodimer with -AT/−ATQ C-terminal ends (ApoAII-2) and 8766-m/z unglycosylated ApoCIII (ApoCIII-0) protein were found to be decreased most significantly in patients with pancreatic cancer, including those with early-stage (stages I and II) disease. These findings indicate that the reduction of these specific forms of apolipoprotein is not a consequence of reduced protein production due to destruction of pancreatic tissue, but is attributable to certain post-translational mechanisms.

It has been reported that increased serum carboxyl- and aminopeptidase activities generate cancer-specific peptidome patterns through cleavage of the C- or N-termini of various plasma/serum proteins [24]. Increased serum carboxypeptidase A activity has been reported in patients with early-stage pancreatic cancer [25]. Synthetic *N*-acetyl-phenyl-L-3-thiaphenylamine has been used as a sensitive substrate to measure the activity of the enzyme, but the small initial cleavage of a C-terminal glutamine

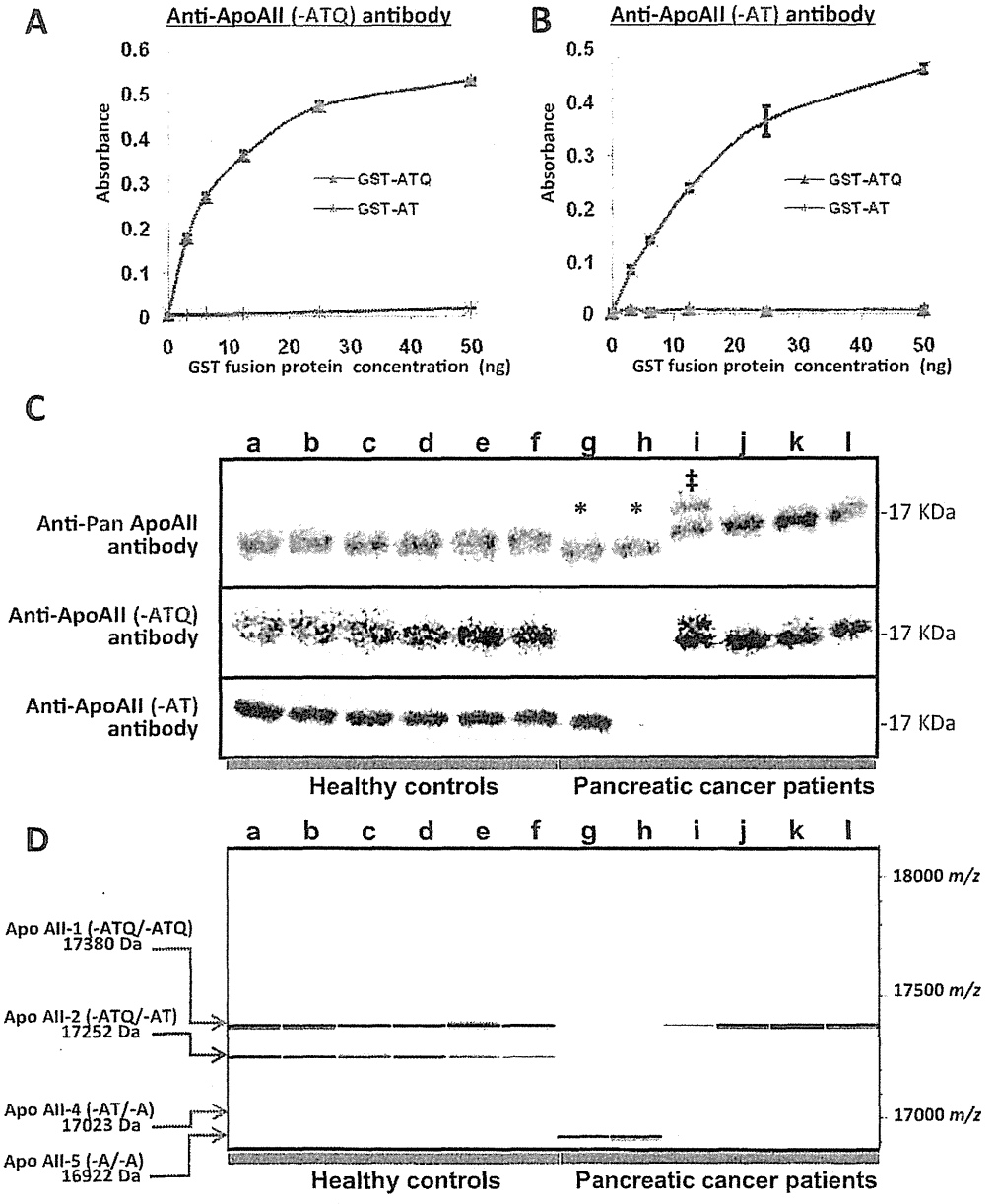

Figure 6. Detection of truncated ApoAII peptide. (**A and B**) Reactivity of anti-ApoAII (-ATQ) (**A**) and anti-ApoAII (-AT) (**B**) peptide antibodies with GST-tagged ApoAII monomers bearing -ATQ (GST-ATQ) (amino acids 1–100) and -AT (GST-AT) (amino acids 1–99) termini. (**C**) Non-denaturing immunoblot analysis of plasma samples from healthy controls (lanes a-f) and representative pancreatic cancer patients (lanes g-l) with antibodies against pan ApoAII protein (top), ApoAII (-ATQ) peptide (middle), and ApoAII (-ATQ) peptide (bottom). A pancreatic cancer patient (lane i) shows a slower-migrating ApoAII protein (indicated by ‡), but its molecular nature remains to be determined. (**D**) Gel-like image of oMALDI-QqTOF-MS spectra within the range 16,900–18,100 m/z for representative healthy controls (lanes a-f corresponding to **C**) and pancreatic cancer patients (lanes g-l corresponding to **C**).
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(Q) residue may prime/sensitize ApoAII protein to further proteolytic digestion, and thus the degradation/decrease of ApoAII-2 might be detectable sensitively even in patients with early-stage pancreatic cancer. The reciprocal increase of the ApoAII homodimer with -A/−A ends (ApoAII-5) supports this notion (Fig. 5A). Some pancreatic cancer patients even showed complete depletion of ApoAII-1 and -2 proteins and the appearance of novel ApoAII-4 and -5 proteins (lanes f and g, Fig. 6C and 6D), indicating that enhancement of proteolytic digestion is responsible for the reduction of ApoAII-2 in these patients.

There is a weakness to be overcome before the current findings can be applied clinically. It is generally accepted that a decrease in the level of any biomarker is difficult to employ as a diagnostic measure, because a highly reproducible measurement system is needed in order to avoid false negativity. The small protein

modifications reported here can currently be detected and quantified only by MS. We constructed a robust analytical system employing automated sample preparation using magnetic beads [26]. This automation reduced fluctuations in handling, and the use of orthogonal MS eradicated any mass inaccuracy caused by the slightly uneven surfaces of MALDI plates. We also carefully eliminated any possible pre-analytical bias of sampling procedures and avoided using complicated computing algorithms. However, MS-based protein quantification is still a complicated technology for routine clinical use, and its application to cancer screening requires further improvement of robustness, throughput, and cost efficiency.

In this study we successfully produced a pair of antibodies specifically recognizing the intact ApoAII peptide with an -ATQ terminus and the truncated ApoAII peptide with a -AT terminus (Fig. 6). We are now in the process of establishing a sandwich enzyme-linked immunosorbent assay to quantify the ApoAII-2 heterodimer with a hemi-truncated C-terminus (-ATQ/−AT) using these antibodies. We think that the perfection of this assay will greatly facilitate the clinical applicability of the present findings.

Supporting Information

Figure S1 Decrease of the 17252-m/z peak in patients with pancreatic cancer.
(PDF)

Figure S2 Identification of ApoAII-2 heterodimer.
(PDF)

Figure S3 Identification of ApoAII-2 heterodimer.
(PDF)

Figure S4 Confirmation of protein identity by immunoprecipitation and MS.
(PDF)

Figure S5 Identification of ApoCIII.
(PDF)

Figure S6 Correlation of ApoAII-2 and ApoCIII0.
(PDF)

Table S1 Clinical characteristics of individuals in Cohorts 1 to 3.
(PDF)

Table S2 Clinical characteristics of individuals in Cohort 4 (n = 833).
(PDF)

Table S3 Reduction of plasma ApoAII-2 and ApoCIII-0 in patients with pancreatic cancer (Cohorts 1 and 2).
(PDF)

Table S4 Validation of ApoAII-2 and ApoCIII-0 in the German cohort (Cohort 3).
(PDF)

Table S5 Reduction of ApoAII-2+ApoCIII-0 in patients with early-stage pancreatic cancer (Cohort 4).
(PDF)

Methods S1
(PDF)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: KH TY. Performed the experiments: KH YO NO. Analyzed the data: KH HK T. Sakuma M. Shitashige. Contributed reagents/materials/analysis tools: TO KF SN NS HN TI AT T. Shimahara M. Shimahara YY TK MWB. Wrote the paper: KH TY.

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RESEARCH ARTICLE Open Access

Salvage chemoradiotherapy after primary chemotherapy for locally advanced pancreatic cancer: a single-institution retrospective analysis

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Abstract

Background: There is no consensus on the indication for salvage chemoradiotherapy (CRT) after failure of primary chemotherapy for locally advanced pancreatic cancer (LAPC). Here we report on the retrospective analysis of patients who received salvage CRT after primary chemotherapy for LAPC. The primary objective of this study was to evaluate the efficacy and safety of salvage CRT after primary chemotherapy for LAPC.

Methods: Thirty patients who underwent salvage CRT, after the failure of primary chemotherapy for LAPC, were retrospectively enrolled from 2004 to 2011 at the authors' institution. All the patients had histologically confirmed pancreatic adenocarcinoma.

Results: Primary chemotherapy was continued until progression or emergence of unacceptable toxicity. Eventually, 26 patients (87%) discontinued primary chemotherapy because of local tumor progression, whereas four patients (13%) discontinued chemotherapy because of interstitial pneumonitis caused by gemcitabine. After a median period of 7.9 months from starting chemotherapy, 30 patients underwent salvage CRT combined with either S-1 or 5-FU. Toxicities were generally mild and self-limiting. Median survival time (MST) from the start of salvage CRT was 8.8 months. The 6 month, 1-year and 2-year survival rates from the start of CRT were 77%, 33% and 26%, respectively. Multivariate analysis revealed that a lower pre-CRT serum CA 19–9 level (≤ 1000 U/ml; $p = 0.009$) and a single regimen of primary chemotherapy ($p = 0.004$) were independent prognostic factors for survival after salvage CRT. The MST for the entire patient population from the start of primary chemotherapy was 17.8 months, with 2- and 3-year overall survival rates of 39% and 22%, respectively.

Conclusions: CRT had moderate anti-tumor activity and an acceptable toxicity profile in patients with LAPC, even after failure of gemcitabine-based primary chemotherapy. If there are any signs of failure of primary chemotherapy without distant metastasis, salvage CRT could be a treatment of choice as a second-line therapy. Patients with relatively low serum CA19-9 levels after primary chemotherapy may achieve higher survival rates after salvage CRT. The strategy of using chemotherapy alone as a primary treatment for LAPC, followed-by CRT with salvage intent should be further investigated in prospective clinical trials.

Trial registration: 2011–136

Keywords: Pancreatic cancer, Locally advanced pancreatic cancer, Induction chemotherapy, Salvage therapy, Chemoradiotherapy, Prognostic factor

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Background

The prognosis of pancreatic cancer remains dismal. The 5-year overall survival of patients with pancreatic cancer is < 5%. In Japan, about 27,000 patients are estimated to have pancreatic cancer, and almost the same numbers of deaths annually are attributable to this cancer. Although surgical resection offers the opportunity for cure, less than 20% of patients are diagnosed with pancreatic cancer at an early resectable stage. At initial diagnosis, ≥ 80% of patients with pancreatic cancer have locally advanced or metastatic disease.

Locally advanced pancreatic cancer (LAPC) is defined as surgically unresectable disease without detectable metastases. Historically, concurrent chemoradiotherapy (CRT) with 5-fluorouracil (5-FU) has been the standard treatment since it offers survival benefit when compared with best supportive care [1], radiotherapy alone [2] and chemotherapy with 5-FU alone [3]. Recently, 5-FU has been replaced by oral fluorouracil analogues such as S-1 in East Asia [4] and capecitabine in Western countries. When taken orally these drugs are much more convenient to administer than 5-FU, which usually requires protracted venous infusion. S-1 is an oral agent that contains tegafur, gimeracil and oteracil in a molar ratio of 1:0.4:1 [5]. S-1 is reported to be at least equivalent to or even more active than 5-FU when combined with radiotherapy for LAPC [6-8].

The standard method used for the detection of metastases from pancreatic cancer is computed tomography (CT). Several investigators have reported that intraoperative staging can reveal occult peritoneal dissemination in 6–37% of the patients with CT-diagnosed LAPC [9-11]. Analysis of patterns of failure after definitive CRT for LAPC has shown that more than half of the patient will have distant metastasis at the first time of failure [12]. Because radiotherapy involving the primary site offers little benefit to patients with occult distant metastasis, increasingly more oncologists believe that chemotherapy would be a preferable initial therapeutic approach for patients with LAPC [13]. During initial chemotherapy, rapidly progressive chemotherapy-resistant distant metastases will present within a few months. After 3–6 months of induction chemotherapy, LAPC that remained local would be an indication for consolidative or salvage CRT. However, there is no consensus on the indications for additional CRT following primary chemotherapy for LAPC, as well as the optimal time period for the administration of primary chemotherapy. Here we report on the results of a retrospective analysis of this strategy, including primary chemotherapy and salvage CRT, for patients with LAPC. The primary objective of our study was to evaluate the efficacy and safety associated with salvage CRT following primary chemotherapy for LAPC. The secondary objective was to elucidate the prognostic factors that affect survival after CRT.

Methods

Patients

Between October 2004 and August 2011, 98 patients who were diagnosed as having LAPC underwent CRT at the author's institution. Sixty-seven patients were excluded from the study because they had received definitive CRT as the first therapeutic modality. One patient was excluded because he had undergone consolidative CRT after primary chemotherapy. The remaining 30 patients underwent salvage CRT after the failure of primary management with chemotherapy alone. All of the patients had histologically confirmed pancreatic adenocarcinoma. They were subjected to intensive analysis. The clinical data from these patients were entered into the database in September 2012. Our institutional review board (Institutional Ethical Review Board of the National Cancer Center) approved this study.

Treatment strategy

At the first diagnosis, multidetector row CT involving the chest and abdomen were performed for the assessment of the local extension of the primary tumor, and for excluding distant metastases. CT based criteria regarding tumor unresectability included encasement or occlusion of the celiac trunk, common hepatic artery, superior mesenteric artery or aorta. All of the patients with obstructive jaundice underwent biliary drainage prior to treatment.

Until December 2007, primary management with CRT combined with 5-FU was the principal treatment of choice for patients with LAPC [14]. Since 2006, several prospective phase II clinical trials involving patients with LAPC were conducted at the authors' institution [4,8,15,16]. CRT combined with S-1 has been regarded as an optional treatment of choice in Japan [7,8]. A multi-institutional phase II trial with gemcitabine (GEM) alone for LAPC yielded promising results with a low toxicity profile [15]. Additionally, our retrospective study revealed that there was no difference in the survival rates of the patients who received CRT or GEM-based chemotherapy alone as a primary therapy for LAPC [17]. Although direct comparison between primary CRT and primary chemotherapy alone has not yet been made in a prospective clinical trial, GEM monotherapy has been regarded as the first treatment of choice in clinical practice since January 2008.

Currently, all of the patients with LAPC are informed of two first-line treatments of choice, namely GEM monotherapy and CRT combined with S-1. If a patient with LAPC has an indication suitable for participation in a clinical trial, the patient will be given additional information about that trial. The patients themselves selected

one of these treatments. The current study included patients who initially entered prospective clinical trials involving primary chemotherapy and who subsequently received CRT as a salvage treatment.

Eligibility criteria for salvage CRT

Indications for salvage CRT following chemotherapy included the following: no distant metastasis; no prior radiotherapy of the upper abdomen; Karnofsky performance status (KPS) ≥ 70; adequate hematologic function (leucocyte count ≥ 3,500/μL and platelet count ≥ 100,000/μL); and hepatic function (bilirubin ≤ 2.0 mg/dL, aspartate aminotransferase (AST)/alanine aminotransferase (ALT) ≤ 150 U/L) and renal function (serum creatinine < 1.5 mg/ml). The exclusion criteria were the presence of: an active gastroduodenal ulcer; watery diarrhea; ascites; active infection; or mental disorder. Written informed consent was obtained from each patient before starting each treatment.

First-line chemotherapy

Primary chemotherapy was continued until disease progression, the emergence of unacceptable toxicity or a patient's refusal of treatment. First-line chemotherapy mostly consisted of GEM alone [Table 1]. GEM was administered intravenously at a dose of 1,000 mg/m^2 over 30 min on days 1, 8 and 15, and was repeated every 4 weeks as one course. Patients with grade 3–4 hematological toxicities underwent dose reduction to 800 mg/m^2 or skipped at least one administration of GEM. Prophylactic granulocyte-colony stimulating factor support was not used.

Chemoradiotherapy

A planning CT was required to determine target volumes on the three-dimensional treatment planning system. A total dose of 50.4 Gy was delivered in 28 fractions using a linear accelerator of energy ≥ 10 MV. The clinical target volume (CTV) included the gross primary tumor and metastatic lymph nodes only. Elective nodal irradiation was not applied in this cohort. The planning target volume (PTV) was defined as the CTV plus 1 cm in all directions and a 1.5-2.0 cm margin in the craniocaudal direction to account for respiratory organ motion. The dose was prescribed to the center of the PTV. Typically, a 4 or 5 field technique was used to minimize high-dose radiation exposure in the surrounding organs.

Radiotherapy was delivered concomitantly with either 5-FU or S-1. Protracted 5-FU infusion was mainly administered until July 2008, and oral S-1 was given thereafter. Concomitant 5-FU was administered as a protracted venous infusion at a dose of 200 mg/m^2/day from days 1–5 each week during the course of radiotherapy [14]. S-1 was administered orally twice daily after breakfast and dinner on weekdays (Monday through Friday) during irradiation. The standard dose of S-1 with concurrent radiotherapy for LAPC was 80 mg/m^2/day [4]. Maintenance chemotherapy with S-1 was indicated for patients without obvious clinical progression during CRT, with sufficient performance status and organ function.

Table 1 Patient characteristics (n = 30)

Characteristic	No. of patients	% patients
Age (years)		
Median (range)	65 (42–81)	
Gender		
Male	16	53
Female	14	47
Karnofsky performance status		
90-100	22	73
70-80	8	27
0-60	0	0
Tumor location		
Head	15	50
Body and Tail	15	50
Nodal status		
Negative	18	60
Positive	12	40
Baseline tumor diameter (cm)		
Median (range)	4.5 (2.1-7.8)	
Baseline serum CA19-9 level (U/ml)		
Median (range)	872 (0–35490)	
≥ 1,000	14	47
100-1,000	11	37
< 100	5	17
Pre-CRT tumor diameter (cm)		
Median (Range)	4.1 (1.9-8.4)	
Pre-CRT serum CA19-9 Level (U/ml)		
Median	631 (0–50440)	
≥ 1,000	11	37
100-1,000	12	40
< 100	7	23
Regimens of primary chemotherapy		
Gemcitabine alone	24	80
Gemcitabine + α	6	20

CRT chemoradiotherapy.

Response and toxicity assessment

All of the medical charts of the eligible patients were reviewed. Information on potential prognostic factors was collected and included: age; gender; performance status; tumor diameter; change in serum carbohydrate

antigen 19–9 (CA19-9) level; and sequence of treatments. Contrast-enhanced CT was performed before starting every two cycles of primary chemotherapy, before and at the end of CRT, and every 2 months after CRT. Objective tumor response was evaluated radiologically according to the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 [18]. CA19-9 was continuously measured once per month. Toxicities were prospectively recorded at each patient's visit using the Common Terminology Criteria for Adverse Events (CTCAE) version 3.0. The highest grades of toxicity observed during CRT and after CRT were recorded.

Statistical analysis

Overall survival from the start of primary chemotherapy and salvage CRT was estimated using the Kaplan-Meier method. Times to progression at the primary tumor site or distant sites were also calculated. Progression was defined as confirmation of progressive disease on CT images using the RECIST criteria. For univariate and multivariate analysis, all of the variables were dichotomized according to clinical relevance based on the previous literature. Univariate analyses were performed using the log-rank test. A Cox's proportional hazards model was developed to identify significant factors influencing survival after CRT. Possible confounded variables were excluded from multivariate analysis. All of the tests of hypotheses were conducted at an alpha level of 0.05 with a 95% confidence interval (CI). All of the statistical analyses were performed using SPSS Statistics version 17.0 (SAS Institute, Tokyo, Japan).

Results

Patient characteristics

Thirty patients with LAPC received primary chemotherapy and salvage CRT. The patient characteristics are summarized in [Table 1]. For first-line chemotherapy, all of the patients received GEM-based chemotherapy. GEM-based chemotherapy included GEM alone in 24 patients (80%) and GEM-based combination chemotherapy in six patients (20%).

Sequel of first-line chemotherapy

The median number of cycles of GEM in 24 patients who received GEM monotherapy was six (range, 1–41). Best tumor response assessed radiologically and best CA19-9 response to first-line chemotherapy are summarized in Table 2. A partial response (PR) was achieved in nine patients, with a response rate of 30%. Among 24 patients whose baseline serum CA19-9 level was >100 U/ml, the median CA19-9 level decreased from 1151 U/ml at baseline to 159 U/ml at minimum during first-line chemotherapy. In these patients, the CA19-9 level decreased by ≥ 50% in 21 patients (88%); the median time to reach the minimum CA19-9 level was 4.0 (range, 1.8-13.0) months. After failure of first-line GEM-based chemotherapy, seven patients (23%) proceeded to second-line chemotherapy with S-1 alone. The median duration of continuing second-line chemotherapy was 3.0 months.

Table 2 Best response to primary chemotherapy

Tumor response	No. of patients	% patients
Radiological response		
Partial response	9	30
Stable disease	19	63
Progressive disease	2	7
CA19-9 response (base line CA19-9 > 100 U/ml)		
≥ 50% decrease	21	88
< 50% decrease	1	4
Increase	2	8

Eventually, 26 patients (87%) discontinued primary chemotherapy because of local tumor progression, whereas four patients (13%) discontinued chemotherapy because of interstitial pneumonitis caused by GEM. The reasons for discontinuation of the primary chemotherapy are summarized in Table 3.

Sequence of salvage CRT

Thirty patients started salvage CRT after the failure of the primary chemotherapy. The median time between the start of the primary chemotherapy and the start of CRT was 7.9 (range, 3.0-37.3) months. All of the patients completed the course of radiotherapy without major interruption. The median duration of CRT was 42 (range, 38–45) days. Administration of the combined chemotherapeutic agents involved protracted infusion of 5-FU in 14 patients (47%) and oral S-1 in 16 patients (53%). Toxicities during and after CRT are listed in Table 4. Hematological toxicity was relatively mild and there was no grade 4 toxicity. The most frequent grade 3 hematological toxicity was leucopenia. Grades 3 and 4

Table 3 The reasons for discontinued primary chemotherapy

Reason	No. of patients	% patients
Presence of any types of primary disease progression (n = 26)		
Enlargement of tumor	14	47
Elevation of tumor marker	7	23
Carcinomatous pain	5	17
Obstructive jaundice	5	17
Duodenal hemorrhage	2	7
Absence of disease progression (n = 4)		
Interstitial pneumonia	4	13