Table 3. Gene ontology pathway analysis based on the Affymetrix's microarray data

Entry ID	Name	Definition	*p*-value
GO0031055	Chromatin remodeling at centromere	Dynamic structural changes in centromeric DNA.	3.13×10^{-4}
GO0031508	Centromeric heterochromatin formation	The assembly of chromatin into heterochromatin near the centromere.	3.13×10^{-4}
GO0006333	Chromatin assembly or disassembly	The formation or destruction of chromatin structures.	5.36×10^{-4}
GO0006346	Methylation-dependent chromatin silencing	Repression of transcription by methylation of DNA, leading to the formation of heterochromatin.	6.26×10^{-4}
GO0010216	Maintenance of DNA methylation	Any process involved in maintaining the methylation state of a nucleotide sequence.	6.26×10^{-4}
GO0034508	Centromere complex assembly	The aggregation, arrangement and bonding together of proteins and centromeric DNA molecules to form a centromeric protein—DNA complex. Includes the formation of the chromatin structures, which form a platform for the kinetochore, and assembly of the kinetochore onto this specialized chromatin. In fission yeast and higher eukaryotes this process also includes the formation of heterochromatin at the outer repeat (pericentric) regions of the centromere.	1.25×10^{-3}
GO0006325	Establishment and/or maintenance of chromatin architecture	The specification, formation and maintenance of the physical structure of eukaryotic chromatin.	2.85×10^{-3}
GO0006342	Chromatin silencing	Repression of transcription by conversion of large regions of DNA into an inaccessible state often called heterochromatin.	3.75×10^{-3}
GO0031507	Heterochromatin formation	The assembly of chromatin into heterochromatin, a compact and highly condensed form that is often, but not always, transcriptionally silent.	3.75×10^{-3}
GO0051276	Chromosome organization and biogenesis	A process that is carried out at the cellular level that results in the formation, arrangement of constituent part or disassembly of chromosomes, structures composed of a very long molecule of DNA and associated proteins that carries hereditary information.	4.45×10^{-3}
GO0045814	Negative regulation of gene expression, epigenetic	Any epigenetic process that stops, prevents or reduces the rate of gene of expression.	4.69×10^{-3}
GO0006306	DNA methylation	The covalent transfer of a methyl group to either N-6 of adenine or C-5 or N-4 of cytosine.	4.69×10^{-3}
GO0006304	DNA modification	The covalent alteration of one or more nucleotide sites in DNA, resulting in a change in its properties.	7.81×10^{-3}
GO0043414	Biopolymer methylation	The covalent attachment of a methyl residue to one or more monomeric units in a polypeptide, polynucleotide, polysaccharide or other biological polymer	0.0115
GO0016569	Covalent chromatin modification	The alteration of DNA or protein in chromatin by the covalent addition or removal of chemical groups.	0.0121
GO0032259	Methylation	The process by which a methyl group is covalently attached to a molecule.	0.0140
GO0040029	Regulation of gene expression, epigenetic	Any process that modulates the frequency, rate or extent of gene expression; the process is mitotically or meiotically heritable, or is stably self-propagated in the cytoplasm of a resting cell and does not entail a change in DNA sequence.	0.0143
GO0006338	Chromatin remodeling	Dynamic structural changes to eukaryotic chromatin occurring throughout the cell division cycle. These changes range from the local changes necessary for transcriptional regulation to global changes necessary for chromosome segregation.	0.0153
GO0031497	Chromatin assembly	The assembly of DNA, histone proteins and other associated proteins into chromatin structure, beginning with the formation of the basic unit, the nucleosome, followed by organization of the nucleosomes into higher order structures, ultimately giving rise to a complex organization of specific domains within the nucleus	0.0260

overexpressed in poorly differentiated neuroblastoma at the protein level recently.[39]

Northern blot analysis revealed that LSD1 expression was hardly detectable in 16 normal tissues except the testis (data not shown). The aberrant overexpression of LSD1 in many tumor types may make it a good candidate as a therapeutic molecular target with minimum side effects. Conversely, it has recently been reported that LSD1 serves as a key regulator of neural stem cell proliferation.[40] This kind of information also indicates that we should carry on the development of novel anticancer therapy with utmost care. To date, synthetic inhibitors of classical histone deacetylases have been widely used as biological tools for epigenetic studies, and some have advanced to clinical studies. In addition, development of histone methyltransferase and demethylase inhibitors has recently been reported.[41,42] Bisguanidine and biguanide, polyamine analogues, may be the potential inhibitors for LSD1-dependent demethylation.[43–45] Unlike JmjC family demethylases, LSD1 mediates an amine oxidase reaction, which reduces molecular oxygen, and generates hydrogen peroxide. The nonselective monoamine oxidase inhibitor, tranylcypromine, which is currently used for the treatment of mental disorders, has been studied as an LSD1 inhibitor, because tranylcypromine generally functions covalently modifying the flavin cofactor.[20,45–47] Although LSD1 inhibitors are still under development, those may have a great potential to develop as anticancer reagents.

The pathway analysis using the cells in which LSD1 expression was knocked down by siRNA indicated that LSD1 could be involved in regulation of a variety of chromatin functions such as chromatin remodeling, heterochromatin formation and DNA dynamics (Table 3). The results have revealed that abnormally high levels of LSD1 expression cause dysregulation of chromatin structure and gene transcription and contribution to malignant transformation of the cells. Our data are consistent with the evidences indicating that the disruption of chromatin remodeling is tightly associated with human cancer.[48,49] Furthermore, our pathway analysis using the KEGG database also confirmed that LSD1 could regulate p53 signal pathway and affected the TGF-beta pathways (Supporting Information Table 3). LSD1 was known to regulate the function of p53 through the demethylation of mono- and dimethyl groups at K370, a site which is monomethylated by SMYD2.[28–30] LSD1 could prevent the accumulation of the dimethyl groups of p53 by demethylating p53K370Me2 and then inhibit the binding of 53BP1 to p53.[28] Likewise, this might be one of the examples how LSD1 could repress p53-mediated transcriptional upregulation and prevent apoptosis and contribute to human carcinogenesis in addition to chromatin modification.

In conclusion, we found that LSD1 was overexpressed in bladder, lung and colorectal cancers, through early to late stages in carcinogenesis. It is present in the nucleus and promotes proliferation possibly through regulation of a wide variety of chromatin functions. Further validation with functional analyses of this protein in the context of human carcinogenesis may assist to development of novel therapeutic strategies for bladder and other tumors.

Acknowledgements

The authors thank Professor Gillian Murphy and the members of her laboratory for substantial technical support. They also thank Ms. Yuka Yamane, Mr. Kazuhiro Maejima, Ms. Miyuki Saito, Ms. Haruka Sawada and Mr. Kazuyuki Hayashi for technical assistance.

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Cancer Letters 305 (2011) 32–39

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Dendritic cells adenovirally-transduced with full-length mesothelin cDNA elicit mesothelin-specific cytotoxicity against pancreatic cancer cell lines *in vitro*

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ARTICLE INFO

Article history:
Received 15 October 2010
Received in revised form 12 February 2011
Accepted 15 February 2011

Keywords:
Mesothelin
Dendritic cells
Adenovirus vector
Cytotoxic T lymphocytes

ABSTRACT

Mesothelin (MSLN) is an attractive candidate as a molecular target for pancreatic cancer immunotherapy. The purpose of this study was to demonstrate that cytotoxic T lymphocytes (CTLs) generated from peripheral blood mononuclear cells (PBMCs) by stimulation with genetically-modified dendritic cells (DCs) expressing MSLN could produce specific anti-tumor immunity against pancreatic cancer cells endogenously expressing MSLN. MSLN-specific CTLs were generated from PBMCs of healthy donors by *in vitro* stimulation with DCs adenovirally-transduced with the full-length MSLN gene (DC-AxCAMSLN). The cytotoxic activity was tested using a 4-h ^{51}Cr-release assay. The pancreatic cancer cell lines (PK1, CfPAC1, AsPC1), a lymphoblastoid cell lines (LCL) transduced with the MSLN gene, and LCL pulsed with MSLN-epitope peptides were used as target cells. MSLN-specific CTLs induced by *in vitro* stimulation with DC-AxCAMSLN killed pancreatic cancer cell lines expressing MSLN in an HLA-restricted fashion. These CTLs also showed cytotoxic activity against autologous LCL pulsed with multiple MSLN-derived epitope peptides. In addition, $CD8^+$ T cells, as well as $CD4^+$ T cells, sorted from these CTLs showed significant production of interferon-γ when stimulated with DC-AxCAMSLN. The *in vitro* stimulation of PBMCs with DCs transduced with the full-length MSLN gene elicited a potent MSLN-specific cytotoxic activity against pancreatic cancer cell lines endogenously expressing MSLN by recognizing multiple MSLN epitopes and activating both $CD8^+$ T cells and $CD4^+$ helper T cells. These results therefore suggest the potential of developing future clinical applications of the vaccines using genetically-modified DCs expressing MSLN.

1. Introduction

Pancreatic cancer has an extremely poor prognosis, with an overall 5-year survival of 5% [1]. Curative surgery for patients with pancreatic cancer significantly improves their prognosis; however the majority of patients with pancreatic cancer are diagnosed at an advanced stage that makes curative resection very difficult [2]. Chemotherapy using gemcitabine is the standard treatment for unresectable pancreatic cancer at present, although its effects are relatively limited [3]. The development of more effective treatment strategies is therefore urgently needed.

Immunotherapy is a novel approach to the management of pancreatic cancer [4]. The clinical potential of various types of vaccines, such as peptide-based vaccines, dendritic cell vaccines, whole tumor cell vaccines, and recombinant viral- or bacterial-vector based vaccines has been demonstrated in early phase clinical trials [5–10]. The immunological and clinical responses in these studies have been promising, however, they are still insufficient for generating significant clinical benefits. Mesothelin

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0304-3835/$ - see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.canlet.2011.02.013

(MSLN), a glycosylphosphatidylinositol-linked cell surface glycoprotein, is overexpressed in pancreatic ductal adenocarcinomas, however, is not expressed in normal tissues except mesothelial cells, which makes it an attractive candidate as a molecular target for pancreatic cancer immunotherapy [11–14]. In fact, several early phase clinical trials targeting MSLN have recently been reported, including a peptide vaccine, a DNA vaccine, a recombinant immunotoxin, and a chimeric anti-MSLN monoclonal antibody, and immunological responses and some minor clinical responses have been reported [15–20].

Dendritic cells (DCs) are potent antigen-presenting cells that play a critical role in the initiation of anti-tumor immune responses [21]. We have previously shown that DCs genetically transduced with the full-length tumor-associated antigen (TAA) are promising for cancer vaccine development [22,23]. This genetically-modified DC vaccine therapy has several advantages, including the fact that delivery of a broad repertoire of both major histocompatibility complex (MHC) class I and class II restricted epitopes offers the possibility for polyvalent immunization and synergistic $CD4^+$ and $CD8^+$ T-cell responses. Our previous studies have demonstrated that DCs adenovirally-transduced with natural TAA such as gp70 and carcinoembryonic antigen (CEA) were effective for inducing TAA-specific cytotoxic T lymphocytes (CTLs) and that they elicited potent anti-tumor responses in mouse models [22,23].

The purpose of this study was to determine the usefulness of DCs adenovirally-transduced with the whole human MSLN gene as a novel vaccine for patients with pancreatic cancer. We investigated whether these genetically-modified DCs expressing MSLN can induce cytotoxic T lymphocytes (CTLs) that show MSLN-specific cytotoxic activity against pancreatic cancer cells endogenously expressing MSLN, while also trying to clarify whether they can simultaneously induce MSLN-specific $CD4^+$ helper T cells *in vitro*.

2. Materials and methods

2.1. Cell lines

The human pancreatic cancer cell lines PK1 (HLA-A24/24), CfPAC1 (HLA-A2/3), and AsPC1 (HLA-A1/26) were purchased from the American Type Culture Collection (Manassas, VA, USA). Autologous Epstein-Barr virus (EBV)-transfected B-lymphoblastoid cells (LCL) were generated from healthy donor peripheral blood mononuclear cells (PBMCs) transformed by EBV, as described previously [24]. The cells were cultured in RPMI-1640 (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Invitrogen Co., Carlsbad, CA), 2 mM L-glutamine (Invitrogen), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen).

2.2. Immunohistochemistry for MSLN

MSLN protein expression was examined by immunohistochemical staining to evaluate the expression pattern of MSLN in 34 consecutive specimens of pancreatic tumors (invasive ductal adenocarcinoma: 10, intraductal papillary mucinous neoplasms (IPMNs) adenoma: 7, carcinoma in situ: 7, invasive adenocarcinoma: 10) that were resected at Wakayama Medical University Hospital. Invasive ductal adenocarcinoma is the most common neoplasm of the pancreas, consisting more than 85% of pancreatic tumors. IPMNs are defined as grossly visible, mucin-producing, predominantly papillary epithelial neoplasms arising from the main pancreatic duct or branch ducts. The intraductal components of IPMNs display broad spectrum of dysplasia ranging from adenoma to carcinoma in situ, and one third of IPMNs have an associated invasive adenocarcinoma and some patients with a non-invasive IPMN subsequently develop invasive ductal adenocarcinoma. Formalin-fixed, paraffin-embedded tissue sections (5 μm) were deparaffinized and rehydrated. Antigen retrieval was performed in 10 mM of sodium citrate buffer (pH 6.0) heated at 121 °C in a steamer for 7 min. The endogenous peroxidase activity was suppressed by a solution of 3% hydrogen peroxide in methanol for 5 min. After being rinsed in Tris-buffered saline (TBS), the sections were incubated with a blocking reagent: Protein block (Dako, Kyoto, Japan) for 20 min at room temperature. The sections were incubated overnight at 4 °C with the primary antibody, a 1:20 dilution of a mouse monoclonal antibody to MSLN (Clone 5B2; LAB VISION, Fremont CA, USA). After rinsing in TBS, the primary antibody was visualized using the Histofine Simple Stain PO kit (Nichirei, Tokyo, Japan) according to the manufacturer's instruction manual. The sections were developed in DAB at room temperature, and counterstained with Mayer's hematoxylin. The immunolabeling of >10% of the neoplastic cells was defined as positive.

2.3. Generation of human DCs

Monocyte-derived DCs were used as antigen-presenting cells to induce CTL responses against MSLN. DCs were generated *in vitro* from the peripheral blood of healthy volunteers. PBMCs isolated from a healthy volunteer's buffy coats using Ficoll-Paque™ PLUS (GE Healthcare, Piscataway, NJ, USA) were separated by adherence to a PRIMARIA™ tissue culture dish (Becton Dickinson) in order to enrich the monocyte fraction. The monocyte-enriched population was cultured for 5 days in AIM-V medium (Invitrogen) containing 2% heat-inactivated autologous serum (AS) supplemented with 1000 U/mL recombinant human granulocyte–macrophage colony-stimulating factor (GM-CSF; R&D Systems, Inc., Minneapolis, MN, USA) and 500 U/mL recombinant human interleukin (rhIL)-4 (kindly provided by Ono Pharmaceutical co., Tokyo, Japan), and then was cultured for additional 24–48 h in the presence of 1000 U/mL rhIL-6 (R&D Systems), 10 ng/mL recombinant human tumor necrosis factor-α (rhTNF-α; R&D Systems), 10 ng/mL rhIL-1β (R&D Systems), and 1 μg/mL prostaglandin E_2 (Sigma–Aldrich) to induce final maturation [25]. The mature DCs were harvested, and the expression of cell surface molecules was analyzed by flow cytometry. Approximately 95% of the cells showed the expression of CD11c, CD80, CD83, and CD86 (data not shown).

2.4. Recombinant adenoviral vector construction

MSLN cDNA, cloned into the pBluescript SK(−) plasmid (provided by Chugai Pharmaceutical co., Ltd., Tokyo, Japan)

was excised by *EcoRI* and *NspI* and blunt-ended, then ligated into the *SwaI* site of cosmid vector pAxCAwt (Takara, Shiga, Japan) to yield pAxCAMSLN. The recombinant adenoviral vector AxCAMSLN, encoding MSLN, was generated by the cosmid-terminal protein complex (COS-TPC) method, as described previously [26]. AxCALacZ encoding β-gal was also generated by the COS-TPC method.

2.5. Gene transduction of DCs

DCs were transfected with AxCAMSLN or AxCALacZ using the centrifugal method [22]. Our previous study showed that the optimal multiplicities of infection (MOIs) was 100 in terms of both the efficiency of transduction and the viability of DCs [27]. Therefore, the MOI for AxCAMSLN was fixed at 100 in this study, and the expression of MSLN on DC-AxCAMSLN was observed to be 61% by flow cytometry, and the viability of DC-AxCAMSLN cells was >90% (data not shown).

2.6. Synthetic peptides

MSLN peptides that bind to HLA-A24 or HLA-A2 molecules at high levels, as described previously [28], were synthesized according to standard solid phase methods, and were purified to >95% purity by high-performance liquid chromatography (Takara). HLA-A24-binding MSLN peptides, FYPGYLCSL ($A24_{(435-443)}$), LYPKARLAF ($A24_{(475-483)}$), and HLA-A2-binding MSLN peptides SLLFLLFSL ($A2_{(20-28)}$), VLPLTVAEV ($A2_{(530-538)}$) were synthesized for the experiments.

2.7. Induction of CTLs from PBMCs

MSLN cDNA-transduced DCs (DC-AxCAMSLN) were irradiated (25 Gy × 3) and transferred into 24-well tissue culture plates (2×10^5 cells/well) and incubated with autologous fresh PBMCs (4×10^6 cells/well) from healthy donors in 1 mL of AIM-V with 2% AS containing rhIL-7 (10 ng/mL; PEPROTECH, Rocky Hill, NJ, USA). RhIL-2 (20 U/mL; PEPROTECH) was added on day 2 in a total volume of 2 mL. On days 7 and 14, the cultures were re-stimulated with DC-AxCAMSLN at a ratio 20:1. Complete medium containing 20 U/mL of rhIL-2 was added every 2–3 days. The cytotoxic activity was analyzed on day 21, after 3 cycles of stimulation.

2.8. Cytotoxicity assay

The cytotoxic activity was tested using a 4-h ^{51}Cr-release assay, as described previously [22]. The percentage of cytotoxic activity was calculated as follows: percentage of lysis = [(cpm of the sample release − cpm of the spontaneous release/(cpm of the maximum release − cpm of the spontaneous release)] × 100. MSLN cDNA-transduced autologous LCL (LCL-AxCAMSLN), LacZ cDNA-transduced LCL (LCL-AxCALacZ), pancreatic cancer cell lines (PK1, CfPAC1, AsPC1), and LCL pulsed with MSLN-epitope peptides (FYPGYLCSL ($A24_{(435-443)}$), LYPKARLAF ($A24_{(475-483)}$), SLLFLLFSL ($A2_{(20-28)}$), and VLPLTVAEV ($A2_{(530-538)}$)) were used as target cells. LCL were pulsed with 20 μg/mL of each MSLN peptides for 16 h at 37 °C.

2.9. Cold target inhibition assay

The antigen specificity of the CTLs induced by the stimulation with genetically-modified DCs expressing MSLN was examined by the cold target inhibition assay. ^{51}Cr-labeled PK1 cells were used as hot targets, and unlabeled LCL-AxCAMSLN or LCL-AxCALacZ were used as cold targets. In a ^{51}Cr-release assay, the effector (CTLs)/hot target (PK1) ratio was fixed at 25, while various cold/hot target ratios were examined.

2.10. Interferon-γ (IFN-γ) release assay

We examined the MSLN-specific $CD4^+$ and $CD8^+$ T-cell responses using an IFN-γ release assay following methods described previously [29]. In brief, $CD4^+$ and $CD8^+$ T cells were isolated from CTLs cultured after 3 cycles of re-stimulation *in vitro* using an autoMACS™ instrument (Miltenyi Biotec, Bergisch Gladbach, Germany). CTLs were washed twice in phosphate-buffered saline containing 0.5% bovine serum albumin (Invitrogen Co., Carlsbad, CA) and 2 mM EDTA. CTLs were then incubated with CD4 or CD8 microbeads (Miltenyi Biotec) for 15 min at 4 °C and then washed prior to separation. Separation was performed using an autoMACS column (Miltenyi Biotec). The column was placed in the magnetic field and magnetically labeled cells were retained in the column and flushed out as positively selected cells when the magnetic field was off. The purity of sorted populations was determined by flow cytometry and was always more than 95% (data not shown). The positively-selected $CD4^+$ and $CD8^+$ T cells (5×10^4) were stimulated with DCs (DC-AxCAMSLN, DC-AxCALacZ, 5×10^3), in a total volume of 200 μL of complete medium in a 96-well round-bottomed plates for 24 h. Thereafter, the supernatants were collected, and the IFN-γ levels were measured using a human IFN-γ Enzyme-linked immunosorbent assay (ELISA) kit (Endogen, Inc., Woburn, MA, USA). Each assay was performed on duplicate samples.

2.11. Statistical analysis

StatView 5.0 software (Abacus Concepts, Inc., Berkeley, CA) was used for all statistical analyses. Statistical analysis was performed by a Student's *t*-test. A *p*-value of <0.05 was considered to be significant.

2.12. The experimental procedures

This experiment was approved by the Committee for Recombinant DNA Experiments of Wakayama Medical University. All experiments were performed in accordance within the Guidelines of this Committee. We obtained written informed consent from all healthy donors before experiments.

3. Results

3.1. Immunohistochemistry of pancreatic tumor tissues

Immunohistochemical analysis was performed to investigate the expression pattern of MSLN in pancreatic tumor tissues (including 10 invasive ductal adenocarcinomas, seven adenomas of IPMNs, seven carcinomas in situ of IPMNs, and 10 invasive carcinomas derived from IPMNs) (Table 1). Positive immunostaining was observed in all 10 cases (100%) of invasive ductal adenocarcinoma, and in seven cases (70%) of invasive carcinomas derived from IPMNs. On the other hand, negative immunostaining was observed in adenomas and carcinomas in situ of IPMNs. Even within the same specimen, the expression of MSLN was observed in the invasive component of IPMNs, but was not detected in the non-invasive component (Fig. 1).

3.2. MSLN expression on target cells

We evaluated the expression of MSLN in LCL-AxCAMSLN, LCL-AxCALacZ and pancreatic cancer cell lines (PK1, CfPAC1, AsPC1) by RT-PCR. We observed strong expression of MSLN in PK1, CfPAC1, AsPC1 and LCL-AxCAMSLN, but not in LCL-AxCALacZ (data not shown).

3.3. MSLN-specific CTL responses induced by MSLN cDNA-transduced DCs

CTLs induced by DC-AxCAMSLN from HLA-A24-positive Donors showed cytotoxic activity against autologous LCL-AxCAMSLN generated from each of the donors, whereas they did not show cytotoxic activity against autologous LCL-AxCALacZ (Fig. 2).

Table 1
MSLN immunohistochemistry summary.

	Invasive ductal adenocarcinoma ($n = 10$)	IPMNs		
		Adenoma ($n = 7$)	Carcinoma in situ ($n = 7$)	Invasive carcinoma ($n = 10$)
Negative	0	7 (100%)	7 (100%)	3 (30%)
Positive	10 (100%)	0	0	7 (70%)

MSLN protein expression was examined by immunohistochemical staining to evaluate the expression pattern of MSLN in 34 consecutive specimens of pancreatic tumors. The immunolabeling of >10% of the neoplastic cells was defined as positive.

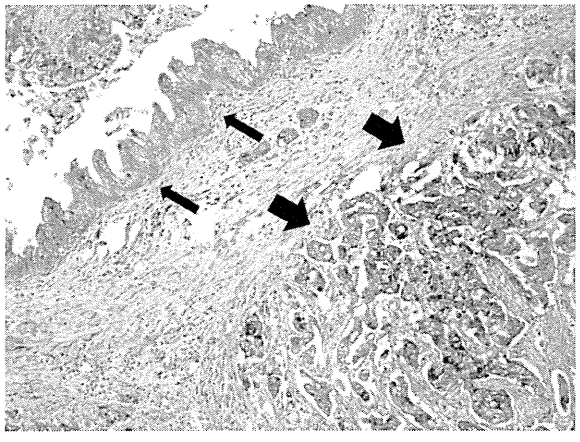

Fig. 1. Immunohistochemical staining for mesothelin protein in the tumor tissue of IPMNs. There is strong labeling of the neoplastic epithelium in the invasive component of IPMNs (thick arrows) but not in the non-invasive component (thin arrows). (magnification 100×).

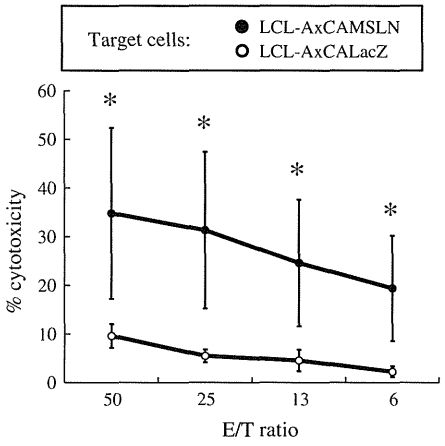

Fig. 2. Cytotoxic activity of CTLs generated from MSLN cDNA-transduced DCs. CTLs cultured after 3 cycles of re-stimulation *in vitro* were used as effectors to test the lysis of autologous LCL-AxCAMSLN and LCL-AxCALacZ. Results were shown as the mean ± SD of seven different donors. *Significant increase of the lysis was shown ($p < 0.01$).

3.4. Cytotoxic activity induced by DC-AxCAMSLN against pancreatic cancer cell lines endogenously expressing MSLN

The CTLs generated by DC-AxCAMSLN from HLA-A24 Donors showed cytotoxic activity against PK1, which endogenously expresses MSLN and possesses the HLA-A24; however they did not show the cytotoxic activity against AsPC1, which endogenously expresses MSLN but does not possess the HLA-A24 (Fig. 3A). The cytotoxic activity against PK1 was suppressed by anti-HLA class I antibody (data not shown). On the other hand, the CTLs generated by DC-AxCAMSLN from HLA-A2 Donors showed cytotoxic activity against CfPAC1 cells, which express MSLN and possess HLA-A2; however, they did not show the cytotoxic activity against AsPC1 cells which endogenously express MSLN but does not possess HLA-A2 nor any shared type of HLA in common with the donors (Fig. 3B). These results suggest that the CTLs induced by genetically-modified DCs expressing MSLN showed MSLN-specific cytotoxic activity that was obviously restricted to the HLA-A types of donors.

3.5. Cold target inhibition assay

To investigate whether the CTL responses against PK1 were dependent on the specificity to MSLN, we carried out a cold target inhibition assay. PK1 cells labeled with $Na_2{}^{51}CrO_4$ were prepared as the hot target, and autologous LCL-AxCAMSLN and LCL-AxCALacZ without labeling were used as the cold targets (inhibitor). The cytotoxic activity of CTLs induced by DC-AxCAMSLN from HLA-A24 positive Donors (Donors 1, 2, and 3) against PK1 was specifically inhibited with the addition of autologous LCL-AxCAMSLN. On the other hand, it was not inhibited by the addition of autologous LCL-AxCALacZ (Fig. 3C).

3.6. MSLN-specific $CD4^+$ and $CD8^+$ T-cell responses induced by DC-AxCAMSLN

To investigate whether MSLN-specific $CD4^+$ and $CD8^+$ T-cell responses in PBMC-derived CTLs were induced by the stimulation with DC-AxCAMSLN, $CD4^+$ T cells and $CD8^+$ T cells were sorted from CTLs, and their ability to produce IFN-γ when they were incubated with DC-AxCAMSLN or DC-AxCALacZ cells was tested. IFN-γ production by the $CD8^+$ T cells incubated with DC-AxCAMSLN was higher than that of $CD8^+$ T cells incubated with DC-AxCALacZ cells. Moreover, IFN-γ production by $CD4^+$ T cells incubated with DC-AxCAMSLN cells was extremely higher than $CD4^+$ T cells incubated with DC-AxCALacZ (Fig. 4).

3.7. The cytotoxic activity induced by DC-AxCAMSLN against autologous LCL pulsed with MSLN-derived epitope peptides

To investigate whether MSLN-derived epitope peptide-specific CTL responses were elicited by the stimulation with DC-AxCAMSLN from PBMCs, the cytotoxic activity of CTLs against epitope peptide-pulsed

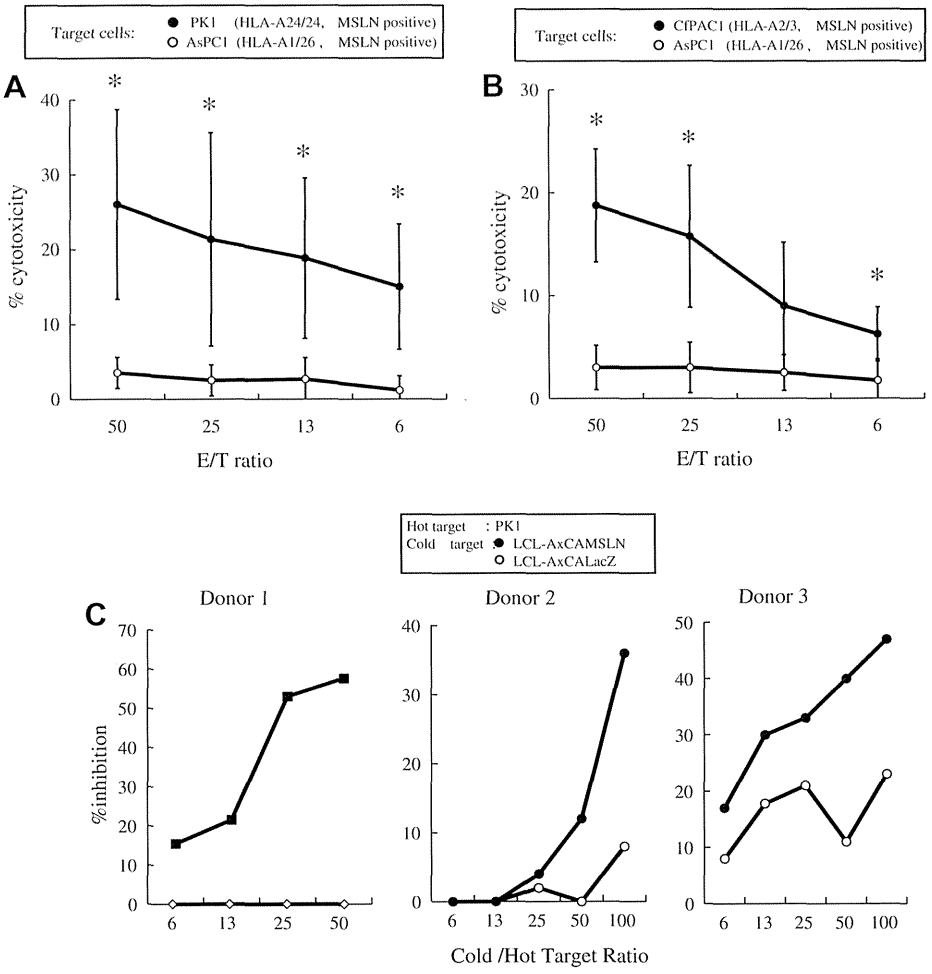

Fig. 3. Cytotoxic activity of CTLs generated from MSLN cDNA-transduced DCs against pancreatic cell lines endogenously expressing MSLN. **A.** Cytotoxic activity against PK1, which endogenously express MSLN and HLA-A24, and against AsPC1, which endogenously express MSLN but not HLA-A24. Results were shown as the mean ± SD of six different donors who possessed HLA-A24. *Significant increase of the lysis was shown ($p < 0.01$). **B.** Cytotoxic activity against CfPAC1, which endogenously express MSLN and HLA-A2, and against AsPC1, which endogenously express MSLN but not HLA-A2. Results were shown as the mean ± SD of four different donors who possessed HLA-A2. *Significant increase of the lysis was shown ($p < 0.05$) **C.** For the cold target inhibition assay, PK1 cells labeled with $Na_2{}^{51}CrO_4$ were prepared as the hot target, whereas MSLN-transduced autologous LCL were used as the cold target (inhibitor). The effector/target (E/T) ratio was fixed at 25.

LCL was examined. CTLs induced by DC-AxCAMSLN from an HLA-A24/A2-positive donor (Donor 1) showed specific lysis against LCL pulsed with MSLN peptides $A24_{(435-443)}$, $A24_{(475-483)}$, $A2_{(20-28)}$, and $A2_{(530-538)}$. CTLs induced by DC-AxCAMSLN from the HLA-A24/26-positive donor (Donor 2) exhibited specific lysis against LCL pulsed with MSLN peptides $A24_{(435-443)}$ and $A24_{(475-483)}$ (Fig. 5). These CTLs showed no cytotoxic activity against LCL that were not pulsed with the peptides.

4. Discussion

In the present study, we first found that CTLs induced by human DCs transduced with full-length MSLN cDNA had strong cytotoxic activity against not only autologous LCL transduced with the MSLN gene, but also pancreatic cancer cell lines naturally expressing MSLN in an HLA-restricted fashion.

In humans, MSLN has been demonstrated to be overexpressed in several cancer types, including pancreatic cancer, ovarian cancer, mesothelioma, lung cancer, uterine serous carcinoma and acute myeloid leukemia, although it is not expressed in normal tissues except mesothelial cells [11,12,14,30–35]. Argani et al. [11] found that MSLN staining was positive in all 60 resected of the primary pancreatic adenocarcinomas they examined, but was negative or only weakly expressed in adjacent normal pancreatic tissues. This finding has been confirmed by many other studies with microarrays, serial analysis of gene expression, and immunohistochemical staining [36–38]. MSLN was also confirmed to be overexpressed in resected invasive ductal adenocarcinomas and invasive carcinomas derived from IPMNs in the present study. Importantly, however, it was not expressed in adenomas and even in carcinoma in situ of IPMNs. Even in the same specimen, the expression of MSLN was observed in the invasive component of IPMNs, but not in the non-invasive components.

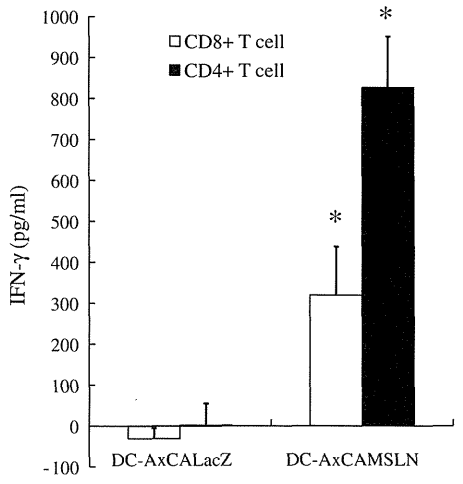

Fig. 4. MSLN-specific $CD4^+$ and $CD8^+$ T-cell responses induced by *in vitro* stimulation using MSLN cDNA-transduced DCs. $CD4^+$ and $CD8^+$ T cells were isolated using an autoMACS system from *in vitro* primed PBMCs after three cycles of re-stimulation by DC-AxCAMSLN cells. Each of the responding lymphocytes populations was stimulated with DC-AxCAMSLN or DC-AxCALacZ in a 96-well round-bottomed plate. Supernatants collected 24 h later were tested for IFN-γ levels. Results were shown as the mean ± SD of five different donors. *Significant increase of IFN-γ levels was shown ($p < 0.01$).

These results suggest that MSLN might have a tendency to be preferentially expressed in invasive tumor cells. In fact, MSLN plays a role in tumor adhesion and dissemination through the interaction between MSLN and MUC-16 [39,40]. MSLN also plays a role of cell proliferation and migration because these processes are inhibited by silencing of MSLN in pancreatic cancer cell lines [13,39]. In addition, normal mesothelial cells express no or little mesothelin protein [30,41,42], and therefore it is expected that MSLN-specific CTLs would not show the cytotoxic activity against them. MSLN is thus suggested to be an ideal target for immunotherapy for patients with pancreatic cancer because of its unique expression pattern, and because of its crucial functions that are closely related to malignant behavior.

With regard to the immunogenicity of MSLN, a clinical study conducted by Jaffee et al. that involved vaccination of pancreatic cancer patients with GM-CSF-transduced pancreatic cancer cell lines showed that 3 of 14 patients developed a post-vaccination delayed-type hypersensitivity (DTH) response to the autologous tumor that was associated with prolonged survival [43]. Interestingly, subsequent immunological studies showed that a strong induction of a $CD8^+$ T cell response to multiple HLA-restricted MSLN epitopes occurred in the 3 patients who had developed a vaccine-induced DTH response [28], thus suggesting that MSLN is strongly immunogenic. In addition, MSLN-specific $CD4^+$ and $CD8^+$ T cells were generated from peripheral lymphocytes of patients with pancreatic cancer in 50% of patients compared with only 20% of healthy individuals according to Johnston et al. [44]. Therefore, our vaccine strategy is expected to elicit MSLN-specific CTLs more effectively in patients with pancreatic cancer than in healthy individuals.

DCs are considered the most potent professional antigen-presenting cells and have the most powerful antigen presenting capacity [45,46]. Therefore, DCs adenovirally-transduced with full-length MSLN cDNA might have potential advantages over other cancer vaccine strategies. In this study, we demonstrated that CTLs induced by *in vitro* stimulation with DC-AxCAMSLN showed specific cytotoxic activity against not only autologous LCL transduced with MSLN cDNA, but also pancreatic cancer cell lines naturally expressing MSLN. In addition, CTLs induced by the stimulation with DC-AxCAMSLN showed specific cytotoxicity against autologous LCL pulsed with the multiple MSLN-derived epitope peptides. These results suggest that CTLs generated by the vaccine using DCs transduced with the full-length MSLN gene might have stronger cytotoxic

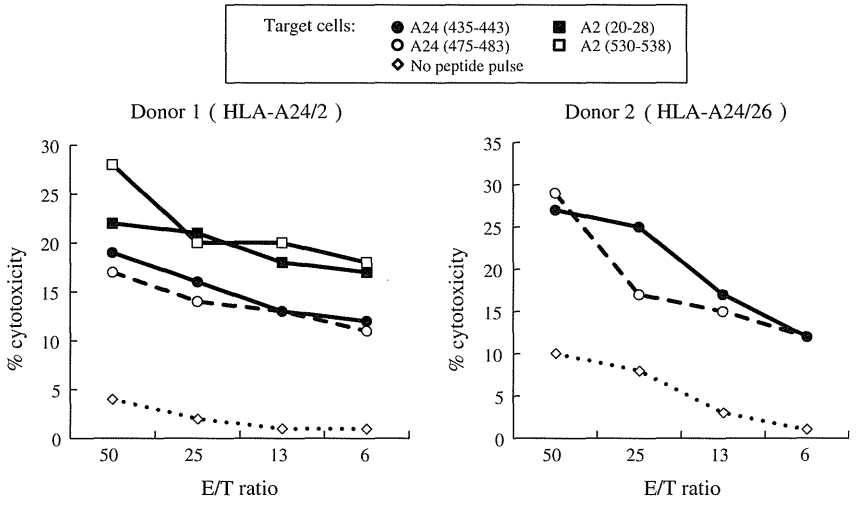

Fig. 5. The cytotoxic activity of CTLs generated from MSLN cDNA-transduced DCs against LCL pulsed with MSLN-epitope peptides. Donor 1 possessed HLA-A24/2 and Donor 2 possessed HLA-A24/26. The autologous LCL were pulsed with various epitope peptides derived from MSLN ($A24_{(435-443)}$, $A24_{(475-483)}$, $A2_{(20-28)}$, and $A2_{(530-538)}$), and were used as target cells.

activity than CTLs generated from one or two epitope peptides derived from MSLN-pulsed DCs, because they could recognize multiple epitopes and also unknown epitopes derived from MSLN.

It is generally accepted that the priming of anti-tumor $CD8^+$ CTLs requires $CD4^+$ T cells [47]. Our previous studies demonstrated that DCs transduced with the TAA gene could elicit tumor-specific $CD4^+$ T cells, and that those $CD4^+$ T cells played a critical role in the priming phase of $CD8^+$ CTLs because the anti-tumor effect was completely abrogated by the depletion of $CD4^+$ T cells in mouse models [23,24,48]. The present study also showed that DC-Ax-CAMSLN activated not only MSLN-specific $CD8^+$ T cells but also $CD4^+$ T cells by the IFN-γ release assay. Therefore, our DC vaccine strategy could more effectively induce MSLN-specific CTLs than the MSLN targeting used in previous studies that was based on single peptide [17,19,49].

In conclusion, MSLN is an ideal immunological target for pancreatic cancer in terms of its expression pattern, crucial cancer-related functions and immunogenicity. The *in vitro*-stimulation of PBMCs with DCs adenovirally-transduced with the entire MSLN gene elicited MSLN-specific cytotoxicity against pancreatic cancer cell lines by inducing the recognition of multiple MSLN epitopes, and activating both $CD8^+$ T cells and $CD4^+$ helper T cells. Therefore, vaccination using these genetically-modified DCs expressing the entire MSLN gene might be promising for clinical applications for patients with pancreatic cancer.

5. Conflicts of Interest

None declared.

Acknowledgements

This study was supported by Grant-in-Aid no.20790964 from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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J Gastrointest Surg (2011) 15:1401–1410
DOI 10.1007/s11605-011-1436-7

ORIGINAL ARTICLE

Influence of Visceral Obesity for Postoperative Pulmonary Complications After Pancreaticoduodenectomy

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Received: 27 December 2010 / Accepted: 26 January 2011 / Published online: 13 May 2011

Abstract
Background We conduct this study to determine whether postoperative complications, including postoperative pulmonary complications (PPCs), are associated with BMI and visceral fat area (VFA) after pancreaticoduodenectomy.
Methods A total of 317 patients undergoing pancreaticoduodenectomy were enrolled. VFA was measured using a cross-sectional computed tomography (CT) scan at the level of the umbilicus by FatScan software version 3.0 (N2 systems Inc., Osaka, Japan). Clinicopathological variables, intraoperative outcomes, and postoperative courses were analyzed.
Results Of all patients, 130 (41.0%) had postoperative complications and PPCs occurred in 14 patients (4.4%). VFA were significantly higher in patients who developed postoperative pancreatic fistula (POPF), PPCs, and mortality than in those patients who did not (P=.0282, P=.0058, and P=.0173, respectively). Multivariate analysis demonstrated that high BMI and high VFA were not independent predictive risk factors for POPF grade B/C and mortality; only high VFA was an independent risk factor influencing PPCs (P=.0390, odds ratio 4.246, 95% confidence interval 1.076–16.759).
Conclusions Visceral obesity was the independent risk factor for the incidence of PPCs after pancreaticoduodenectomy. Preoperative VFA measurement using CT scan is a useful tool for the prediction of the development of PPCs compared to BMI calculation.

Keywords Visceral fat area (VFA) · Body mass index (BMI) · Pancreaticoduodenectomy · Postoperative complications · Postoperative pulmonary complications (PPCs)

Introduction

The prevalence of overweight and obesity is increasing in the general population and reached over 60% in U.S. populations having higher than 25 kg/m^2 body mass index (BMI).[1] Overweight and obesity are associated with numerous complications such as cardiovascular, pulmonary, and metabolic disorders; therefore, surgeons have agreed that generalized obesity is a potential risk factor for operative morbidity and mortality.[2–5] Although BMI is a convenient measure and is useful for assessing the consequence of obesity, it is often unreliable for the evaluation of an individual's status because the proportion and distribution of fat tissue differ greatly from each other. Accordingly, in recent years, excessive visceral fat has been noteworthy in its association with postoperative complications.[6–8]

Postoperative pulmonary complications (PPCs) are common complications after all digestive surgery. Previous reports demonstrated that the incidence of PPCs was 2–13% after pancreaticoduodenectomy,[9–13] which was less than the incidence of postoperative pancreatic fistula (POPF), postpancreatectomy hemorrhage (PPH), and delayed gastric emptying (DGE).

Grant support This work was supported in part by grants from the Japanese Society for Advancement of Surgical Techniques.

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However, one should consider that PPCs are lethal complications frequently requiring extended intensive care, including reintubation and continuous positive airway pressure.

Smetana et al. interestingly reported that BMI was not associated with increased clinical PPCs after surgery in a systematic review.[14] However, obesity is thought to be a risk factor for PPCs and may lead to restrictive pulmonary physiology and further reduction of lung volume.[15, 16] Thus, obesity interrupts the ability to take a deep breath after surgery, and visceral fat (VF) plays an important factor in obesity. Patients with a high volume of VF have high abdominal pressure, resulting in a risk factor for PPCs. Therefore, a new strategy is required for evaluating obesity for the improvement of surgical outcomes after pancreaticoduodenectomy. We conducted this study to determine whether postoperative complications, including PPCs, are associated with BMI and visceral fat area (VFA) after pancreaticoduodenectomy.

Material and Methods

Patients

Between February 2003 and December 2009, 324 consecutive patients underwent pancreaticoduodenectomy in Wakayama Medical University Hospital (WMUH). In the present study, seven patients were excluded because of undergoing hepatopancreaticoduodenectomy (n=3), additional pancreatic tail resection (n=2), total gastrectomy (n=1), or splenectomy (n=1). The 317 enrolled patients in the present study have a median age of 70 years (range 35–91); 181 are male and 136 are female. Patients' characteristics and perioperative and postoperative parameters were reviewed for the following clinical variables: age, gender, concomitant disease, including cardiovascular disease, chronic obstructive pulmonary disease (COPD), and diabetes mellitus, recent smoking history (smoking within 4 weeks prior to surgery),[17] pulmonary function on spirograms (percentage predicted vital capacity, %VC, and the ratio of forced expiratory volume in 1 s to forced vital capacity, FEV1/FVC), preoperative biliary drainage, type of resection (pylorus-preserving pancreaticoduodenectomy, PpPD, or conventional pancreaticoduodenectomy, PD), additional portal vein resection, BMI, VFA, operative time, intraoperative bleeding, red blood cell transfusion, pancreatic texture (soft or hard), and histologic diagnosis (malignant or benign). Informed consents were obtained from all the patients in accordance with the guidelines of the Ethical Committee on Human Research of WMUH.

BMI and Visceral Fat Area

BMI was calculated by patient height and body weight measured preoperatively. The World Health Organization criteria for overweight and obesity were used (overweight, BMI 25.0–29.9; obesity, BMI≥30.0).[18] VFA was measured using a cross-sectional CT scan at the level of the umbilicus by FatScan software version 3.0 (N2 systems Inc., Osaka, Japan),[19] and patients were classified into a high-VFA group (VFA≥130 cm^2) and a low-VFA group (VFA<130 cm^2). In Fig. 1, we show samples in this study population of the amount of visceral fat by FatScan

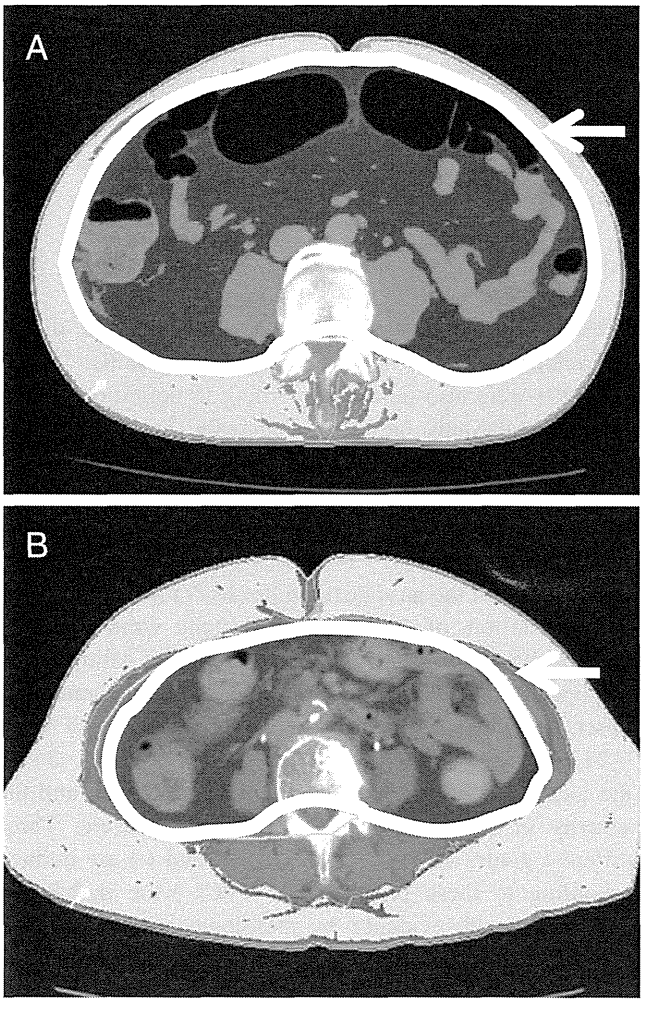

Fig. 1 Distribution of visceral fat area (VFA) by FatScan software on preoperative CT scan. The white line (*white arrow*) outlines the intraperitoneal area. Visceral fat tissue was calculated in the region outlined by the white line by FatScan software. A, VFA was 194.4 cm^2 and body mass index (BMI) was 19.6 kg/m^2, representing high VFA but low BMI. B, VFA was 71.1 cm^2 and BMI was 25.3 kg/m^2, representing low VFA but high BMI (high VFA≥130 cm^2 and low VFA<130 cm^2 and high BMI≥25 kg/m^2 and low BMI<25 kg/m^2)

software on preoperative CT scan. We have no financial relationship to disclose with the FatScan software used.

Surgical Treatment

All patients underwent PpPD with Traverso reconstruction or PD with Child reconstruction, with various extents of lymph node dissection, as described previously.[20, 21] All operations were performed by two experienced pancreatic surgeons (H.Y. and M.T.). Pancreaticojejunostomies were performed with duct-to-mucosa, end-to-side anastomosis in all patients.[22] A 5-French polyethylene pancreatic duct drainage tube (Sumitomo Bakelite, Tokyo, Japan) was used in an external or internal drainage stent for pancreaticojejunostomy. Hepaticojejunal anastomosis was performed end-to-side without stent, followed by pancreaticojejunostomies. Reconstruction of the duodenojejunostomies was performed by the antecolic route.[20] A single prophylactic closed-suction drain was routinely placed in the right upper quadrant around the pancreatic and biliary anastomosis.

Postoperative Complications

POPFs were defined according to the definition of the International Study Group on Pancreatic Fistula.[23] DGE and PPH were also defined according to definitions of the International Study Group of Pancreatic Surgery.[24, 25] In detail, POPF was defined as a drain output of any measurable volume of fluid on or after postoperative day 3 with an amylase content greater than three times the serum amylase activity.[23] DGE was defined by the need for maintenance of the nasogastric tube (NGT) for 3 days, need for reinsertion of NGT for persistent vomiting after postoperative day 3, or inability to tolerate a solid diet by postoperative day 7.[24] PPH is defined by three parameters: onset, location, and severity. The onset is either early (≤24 h after the end of the index operation) or late (<24 h), the location is either intraluminal or extraluminal, and the severity of bleed may be either mild or severe. Three different grades of PPH (grades A, B, and C) are defined according to these parameters.[25] PPCs were defined as pneumonia with evidence by radiologic pulmonary infiltrates and/or the presence of pathogenic bacteria in the sputum culture, and pulmonary atelectasis required frequent bronchoscopic toilet or prolonged ventilator support.[26] Pulmonary edema, pulmonary embolus, and acute respiratory distress syndrome were excluded, similar to previous reports.[27] Other postoperative complications were assessed according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0 (NCI CTCAE v.3.0). In this study, adverse events of grades 2–5 within 30 days after surgery were expediently judged as postoperative complications. Adverse events of grade 1 were excluded because no medical treatment was required. Mortality was defined as death within 30 days after surgery.

Statistical Analysis

Any significance in the correlation between all variables and two variables (BMI and VFA) was evaluated by Mann–Whitney U test and linear regression analysis, where applicable. A P value of less than 0.05 was considered statistically significant. Risk factors for complications were analyzed by logistic regression analysis. Multivariate logistic regression analysis was performed incorporating all factors with $P<0.20$ on univariate analysis. All analyses were performed with Statistical Package for the Social Sciences (SPSS) version 13.0 (SPSS, Chicago, Illinois).

Results

Clinicopathological Characteristics

Table 1 shows the clinicopathological characteristics of patients. Median BMI and VFA were 21.5 kg/m^2 (range 14.8–33.5) and 77.5 cm^2 (range 9.2–261.2), respectively. Association between characteristics and BMI/VFA were summarized in Table 2. BMI was significantly lower in patients with %VC<80 on spirogram (P=.0429) and was higher in patients of benign disease than malignant disease (P=.0334). Otherwise, gender and diabetes mellitus were demonstrated to have significant differences in VFA (P <.0001 and P=.0177, respectively). Other variables were statistically identical with both BMI and VFA.

Surgical Outcome

Median operative time was 358 min (range 185–723) and median estimated blood loss was 735 ml (range 45–9,100; Table 1). Relations between intraoperative outcome and BMI/VFA are shown in Table 3. The linear relationship was demonstrated to have a significant correlation between operative time and both BMI and VFA (P=.0088 and P<.0001, respectively). Estimated blood loss and required red blood cell transfusion were also demonstrated to have a significant correlation with VFA (P=.0008 and P=.0276), whereas for BMI the relation of estimated blood loss was only marginally correlated (P=.0575), and there was no difference between blood transfusion and BMI (P=.3341).

Table 1 Clinicopathological characteristics and intraoperative outcome of patients after pancreaticoduodenectomy

Variable	*N*=317
Age (years), median (range)	70 (35–91)
Gender (male/female)	181/136
Concomitant disease (yes/no)	
Cardiovascular disease	21/296
COPD	15/302
Diabetes mellitus	98/219
Recent smoking history[a] (yes/no)	87/230
Pulmonary function on spirogram	
%VC<80 (yes/no)	31/286
FEV1/FVC ratio<0.70 (yes/no)	64/253
Preoperative biliary drainage (yes/no)	127/190
Type of resection (PpPD/PD)	269/48
Additional portal vein resection (yes/no)	43/274
Pancreatic texture (soft/hard)	137/180
Histologic diagnosis (malignant/benign)	232/85
BMI median (range)	21.5 (14.8–33.5)
BMI (≥25/<25)	46/271
VFA median (range)	77.5 (9.2–261.2)
VFA (≥130/<130)	60/257
Operative time (min), median (range)	358 (185–723)
Estimated blood loss (ml), median (range)	735 (45–9100)
Red blood cell transfusion (yes/no)	114/203

COPD chronic obstructive pulmonary disease, *%VC* percentage predicted vital capacity, *FEV1/FVC ratio* ratio of forced expiratory volume in 1 s to forced vital capacity, *PpPD* pylorus-preserving pancreaticoduodenectomy, *PD* pancreaticoduodenectomy, *BMI* body mass index, *VFA* visceral fat area

[a] Recent smoking history means smoking within 4 weeks prior to surgery[17]

Postoperative Complications

Of all patients, 130 patients had postoperative complications (41.0%). The complications are listed in Table 4. The most common postoperative complication was POPF in 92 patients (29.0%), consisting of 61 grade A (19.2%), 27 grade B (8.5%), and four grade C (1.3%). The incidence of DGE was 35 patients (11.0%), consisting of six grade A (1.9%), ten grade B (3.2%), and 15 grade C (4.7%), and the incidence of PPH was 11 patients (3.5%), consisting of six grade B (1.9%) and five grade C (1.6%). PPCs occurred in 14 patients (4.4%). In the 14 patients with PPCs, seven were unexpectedly admitted to the intensive care unit, and the other five patients required intensive therapy by continuous positive airway pressure in the surgical ward. Mortality occurred in five patients (1.6%); causes were extraluminal hemorrhage (2), sepsis (1), disseminated intravascular coagulation (1), and nonobstructive mesenteric ischemia (1).

Differences Between Postoperative Complications and BMI/VFA

Table 5 shows the statistical differences between the occurrence of postoperative complications and variables of BMI/VFA. Both BMI and VFA in patients with complications were significantly higher than in those without complications (P=.0434 and P=.0189, respectively). Regarding POPF, BMI and VFA demonstrated no significant differences; however, the patients who developed grade B/C POPF had higher VFA than the patients who did not develop POPF (P=.0282). BMI and VFA were demonstrated

Table 2 Association between clinicopathological characteristics and BMI/VFA

Variable	BMI (median, range)	*P* value	VFA (median, range)	*P* value
Age, years (≥70/<70)	21.5 (15.4–33.5)/21.6 (14.8–30.5)	.8392	81.5 (9.2–261.2)/75.7 (10.4–241.1)	.2856
Gender (male/female)	21.5 (15.4–31.2)/21.5 (14.8–33.5)	.6235	93.5 (10.9–261.2)/68.8 (9.2–201.0)	<.0001
Concomitant disease (yes/no)				
Cardiovascular disease	21.1 (15.4–31.2)/21.5 (14.8–33.5)	.7506	81.3 (16.5–183.3)/76.2 (9.2–261.2)	.2040
COPD	21.2 (15.4–26.1)/21.5 (14.8–33.5)	.1443	88.1 (16.5–144.2)/77.1 (9.2–261.2)	.9290
Diabetes mellitus	21.5 (15.8–33.5)/21.5 (14.8–30.0)	.3623	90.6 (10.4–261.2)/75.8 (9.2–230.9)	.0177
Recent smoking history (yes/no)	21.3 (15.8–31.2)/21.6 (14.8–33.5)	.9967	86.1 (18.5–241.1)/75.2 (9.2–261.2)	.1201
Pulmonary function on spirogram				
%VC<80 (yes/no)	21.0 (15.4–26.7)/21.6 (14.8–33.5)	.0429	89.4 (16.5–241.1)/77.0 (9.2–261.2)	.5670
FEV1/FVC ratio<0.70 (yes/no)	21.6 (14.8–30.5)/21.5 (15.4–33.5)	.2694	75.9 (16.0–252.0)/78.8 (9.2–261.2)	.8427
Preoperative biliary drainage (yes/no)	21.4 (15.8–30.5)/21.6 (14.8–33.5)	.7919	74.5 (10.4–241.1)/81.7 (9.2–261.2)	.5363
Type of resection (PpPD/PD)	21.6 (14.8–33.5)/20.6 (15.4–31.2)	.0731	77.5 (10.0–261.2)/78.2 (9.2–209.3)	.3410
Additional portal vein resection (yes/no)	21.4 (16.2–28.3)/21.5 (14.8–33.5)	.8756	84.7 (10.4–208.5)/76.2 (9.2–261.2)	.7399
Pancreatic texture (soft/hard)	21.6 (14.8–31.2)/21.4 (15.4–33.5)	.4697	76.5 (10.0–241.1)/78.9 (9.2–261.2)	.6291
Histologic diagnosis (malignant/benign)	21.2 (15.4–33.5)/22.4 (14.8–30.0)	.0334	75.5 (10.4–252.0)/87.4 (9.2–261.2)	.1853

Table 3 Relation with intraoperative outcome

Variable	BMI			VFA		
	Regression coefficient	R^2	*P* value	Regression coefficient	R^2	*P* value
Operative time (min)	3.878	.022	.0088	0.377	.054	<.0001
Estimated blood loss (ml)	65.417	.011	.0575	7.023	.035	.0008
Red blood cell transfusion (units)	.088	.003	.3341	.012	.015	.0276

BMI body mass index, *VFA* visceral fat area

to have no statistical differences regardless of the occurrences of DGE and PPH. Median VFA of patients with PPCs was 135.7 (range 49.2–174.3), significantly higher than patients without PPCs (75.9; range 9.2–261.2) (P=.0058), whereas there was no difference in BMI. Both BMI and VFA showed no significant differences regardless of the occurrence of the other complications including intra-abdominal abscess, cardiovascular complication, bile leakage, sepsis, bowel obstruction, and wound infection. The mortality group had significantly higher BMI and VFA compared with the nonmortality group (P=.0143 and P=.0173, respectively).

Risk Factors Influencing the Incidence of POPF Grade B/C

BMI and VFA were categorized into two groups and assessed; 46 patients (14.5%) were categorized to high BMI (≥25.0 kg/m^2) and the other 271 patients (85.5%) were categorized to low BMI (<25.0 kg/m^2); similarly, 60 (18.9%) patients were categorized into high VFA (≥130 cm^2) and the other 257 (81.1%) into low VFA (<130 cm^2). Of all 31 patients with POPF grade B/C, five patients were in the high-BMI and high-VFA groups; however, two patients were in only the high-BMI group and two patients were in only the high-VFA group. Multivariate analysis demonstrated that preoperative biliary drainage and soft pancreas predicted the independent risk factor for POPF grade B/C (P=.0407 and P=.0004, respectively; Table 6).

Table 4 Incidence of postoperative complications after pancreaticoduodenectomy

Complication	Number of patients	%
Overall complications	130	41.0
POPF		
All grades	92	29.0
Grade A/B/C	61/27/4	19.2/8.5/1.3
DGE	32	10.1
PPH	11	3.5
Extraluminal hemorrhage	5	1.6
Intraluminal hemorrhage	6	1.9
Intra-abdominal abscess	38	12.0
PPCs	14	4.4
Cardiovascular complication	10	3.2
Bile leakage	9	2.8
Sepsis	11	3.5
Bowel obstruction	10	3.2
Wound infection	19	6.0
Mortality	5	1.6

POPF postoperative pancreatic fistula, *DGE* delayed gastric empting, *PPH* postpancreatectomy hemorrhage, *PPCs* postoperative pulmonary complications

Risk Factors Influencing the Incidence of PPCs

In the 14 patients who developed PPCs, four patients were in the high-BMI group (8.7%). On the other hand, eight patients were in the high-VFA group (13.3%). Six parameters that had a P value ≤ 0.20 by univariate analysis were selected for multivariate analysis, gender, additional portal vein resection, high BMI, high VFA, operative time, and estimated blood loss, and only high VFA was predicted as an independent risk factor influencing the incidence of PPCs (P=.0390, odds ratio 4.246, 95% confidence interval 1.076–16.759; Table 7).

Risk Factors Influencing Mortality

There were five postoperative mortalities (1.6%). Three patients were in the high-BMI group and two were in the low-BMI group, whereas four patients were in the high-VFA group and one was in the low-VFA group. Multivariate analysis demonstrated that recent smoking history (smoking within 4 weeks prior to surgery) was the only independent predictive factor (P=.0321, odds ratio 28.954, 95% confidence interval 1.332–629.405), although high BMI and high VFA were not consequently risk factors for postoperative mortality (P=.1145 and P=.7514, respectively; Table 8).

Discussion

In this study, VFA was demonstrated to be the independent risk factor for the incidence of PPCs after pancreaticoduo-

Table 5 Difference of BMI and VFA in postoperative complications

Complication	BMI (median, range)			VFA (median, range)		
	(+)	(−)	*P* value	(+)	(−)	*P* value
Overall complications	21.9 (15.6–33.5)	21.3 (14.8–31.2)	.0434	86.8 (15.0–261.2)	73.0 (9.2–252.0)	.0178
POPF						
All grades	21.7 (15.8–29.8)	21.4 (14.8–33.5)	.1364	87.7 (9.2–261.2)	74.0 (10.0–252.0)	.1420
Grade B/C	22.2 (16.2–29.8)	21.5 (14.8–33.5)	.0814	93.5 (20.1–261.2)	75.2 (9.2–252.0)	.0282
DGE	21.5 (15.6–28.4)	21.5 (14.8–33.5)	.7626	93.1 (22.3–201.0)	75.9 (9.2–261.2)	.1895
PPH						
Extraluminal hemorrhage	23.0 (19.4–26.6)	21.5 (14.8–33.5)	.3934	93.5 (73.5–160.9)	76.6 (9.2–261.2)	.2498
Intraluminal hemorrhage	24.9 (15.8–28.4)	21.5 (14.8–33.5)	.0507	144.6 (19.9–208.5)	76.6 (9.2–261.2)	.1219
Intra-abdominal abscess	22.2 (15.8–29.8)	21.5 (14.8–33.5)	.3191	89.6 (19.4–261.2)	75.8 (9.2–252.0)	.1088
PPCs	21.6 (19.6–28.3)	21.5 (14.8–33.5)	.1668	135.7 (49.2–174.3)	75.9 (9.2–261.2)	.0058
Cardiovascular complication	20.8 (18.7–28.6)	21.5 (14.8–33.5)	.9972	61.1 (28.7–261.2)	78.3 (9.2–252.0)	.9650
Bile leakage	19.3 (16.8–29.8)	21.5 (14.8–33.5)	.2588	79.3 (19.4–209.3)	77.1 (9.2–261.2)	.5888
Sepsis	20.6 (19.3–30.5)	21.5 (14.8–33.5)	.9306	114.2 (49.6–162.8)	76.2 (9.2–261.2)	.0680
Bowel obstruction	21.4 (19.6–28.6)	21.5 (14.8–33.5)	.6650	78.7 (18.8–261.2)	77.5 (9.2–252.0)	.6727
Wound infection	21.9 (16.8–33.5)	21.5 (14.8–31.2)	.4235	81.2 (15.0–194.8)	76.6 (9.2–261.2)	.8323
Mortality	25.3 (22.6–30.5)	21.5 (14.8–33.5)	.0143	142.7 (73.5–208.5)	76.6 (9.2–261.2)	.0173

Table 6 Univariate and multivariate analyses of risk factors for POPF grade B/C

Variable	Univariate analysis		Multivariate analysis	
	P value	Odds ratio (95% CI)	*P* value	Odds ratio (95% CI)
Age, years (≥70 or <70)	.4370	1.346 (0.636–2.851)	–	–
Gender (male/female)	.6199	1.212 (0.567–2.591)	–	–
Concomitant disease (yes/no)				
Cardiovascular disease	.4353	0.443 (0.057–3.422)	–	–
COPD	.7067	1.236 (0.410–3.720)	–	–
Diabetes mellitus	.1490	0.506 (0.201–1.276)	.4037	0.661 (0.251–1.746)
Recent smoking history[a] (yes/no)	.1447	0.478 (0.178–1.289)	.1291	0.451 (0.161–1.261)
Pulmonary function on spirogram				
%VC<80% (yes/no)	.5154	0.611 (0.139–2.695)	–	–
FEV1/FVC ratio<0.70 (yes/no)	.7272	1.172 (0.481–2.855)	–	–
Preoperative biliary drainage (yes/no)	.0348	2.248 (1.060–4.771)	.0407	2.292 (1.036–5.071)
Type of resection (PpPD/PD)	.3771	1.743 (0.508–5.978)	–	–
Additional portal vein resection (yes/no)	.9099	0.938 (0.311–2.828)	–	–
Pancreatic texture (soft/hard)	.0002	5.296 (2.208–12.703)	.0004	5.100 (2.084–12.481)
Histologic diagnosis (malignant/benign)	.9263	0.975 (0.564–1.683)	–	–
BMI (≥25 or <25)	.1849	1.847 (0.746–4.576)	.0685	2.508 (0.932–6.743)
VFA (≥130 or <130)	.5854	1.282 (0.525–3.133)	–	–
Operative time (min; ≥350 or <350)	.5205	1.283 (0.600–2.741)	–	–
Intraoperative bleeding (ml; ≥1,000 or <1,000)	.7384	1.141 (0.525–2.481)	–	–
Red blood cell transfusion (yes/no)	.9534	0.977 (0.450–2.120)	–	–

COPD chronic obstructive pulmonary disease, *%VC* percentage predicted vital capacity, *FEV1/FVC ratio* ratio of forced expiratory volume in 1 s to forced vital capacity, *PpPD* pylorus-preserving pancreaticoduodenectomy, *PD* pancreaticoduodenectomy, *BMI* body mass index, *VFA* visceral fat area, *CI* confidence interval

[a] Recent smoking history = smoking within 4 weeks prior to surgery[17]

Table 7 Univariate and multivariate analyses of risk factors for PPCs

Variable	Univariate analysis		Multivariate analysis	
	P value	Odds ratio (95% CI)	*P* value	Odds ratio (95% CI)
Age, years (≥70 or <70)	.3300	1.742 (0.570–5.318)	–	–
Gender (male/female)	.0435	4.757 (1.047–21.625)	.1863	2.956 (0.592–14.750)
Concomitant disease (yes/no)				
Cardiovascular disease	.9365	1.088 (0.135–8.748)	–	
COPD	.6666	1.588 (0.194–13.015)	–	–
Diabetes mellitus	.4367	0.597 (0.163–2.190)	–	–
Recent smoking history[a] (yes/no)	.4808	1.497 (0.487–4.600)	–	–
Pulmonary function on spirogram				
%VC<80% (yes/no)	.7354	0.700 (0.088–5.541)	–	–
FEV1/FVC ratio<0.70 (yes/no)	.5763	0.648 (0.141–2.971)	–	–
Preoperative biliary drainage (yes/no)	.7345	0.824 (0.270–2.519)	–	–
Type of resection (PpPD/PD)	.5053	0.640 (0.172–2.383)	–	–
Additional portal vein resection (yes/no)	.1059	2.708 (0.810–9.057)	.2929	2.034 (0.542–7.635)
Pancreatic texture (soft/hard)	.2712	0.515 (0.158–1.679)	–	–
Histologic diagnosis (malignant/benign)	.4451	0.646 (0.210–1.985)	–	–
BMI (≥25 or <25)	.1384	2.486 (0.745–8.291)	.8179	0.841 (0.193–3.673)
VFA (≥130 or <130)	.0009	6.436 (2.142–19.333)	.0390	4.246 (1.076–16.759)
Operative time (min; ≥350 or <350)	.0216	11.018 (1.423–85.304)	.0678	7.258 (0.865–60.932)
Intraoperative bleeding (ml; ≥1,000 or <1,000)	.1697	2.124 (0.725–6.224)	.6801	0.778 (0.236–2.566)
Red blood cell transfusion (yes/no)	.2692	1.832 (0.626–5.361)	–	–

CI confidence interval, *COPD* chronic obstructive pulmonary disease, *%VC* percentage predicted vital capacity, *FEV1/FVC ratio* ratio of forced expiratory volume in 1 s to forced vital capacity, *PpPD* pylorus-preserving pancreaticoduodenectomy, *PD* pancreaticoduodenectomy, *BMI* body mass index, *VFA* visceral fat area

[a] Recent smoking history = smoking within 4 weeks prior to surgery[17]

denectomy. Additionally, BMI did not statistically correlate with the incidence of PPCs. Smetana et al. concluded that obesity was not a risk factor for PPCs[14] because many studies reported that obesity had not increased the risk for PPCs after noncardiothoracic surgery.[28, 29] However, obesity was defined by BMI. As shown in Fig. 1, visceral fat has an individual distribution. In the high-VFA group, 29 patients (48.3%) were interestingly of normal BMI, and 15 patients of high BMI (32.6%) had VFA less than 130 cm^2; these results indicate that BMI and VFA are independent factors from each other for evaluation of the obesity status, and BMI could not always reflect the amount of visceral fat for surgeons.

After abdominal surgery, various factors have been considered to modify postoperative pulmonary dysfunction, that is, rapid shallow breathing, prolonged supine position,[30] pain and anesthesia-induced diaphragmatic dysfunction,[31] and impaired mucociliary clearance.[32] Visceral fat accumulation increases intra-abdominal pressure[33] to pump the diaphragmatic muscle upward, compressing the parenchyma of the lung. Consequently, patients with visceral obesity are affected by a restrictive respiratory impairment with decreased expiratory reserve volume and functional residual capacity.[15, 16] It was considered that the restrictive respiratory impairment caused by visceral fat accumulation may further impair pulmonary function in the perioperative period and lead to PPCs.

In the present study, overall POPF were not associated with BMI and VFA; however, the patients who developed POPF grade B/C showed significantly higher VFA than patients without grade B/C POPF, regardless of risk factors for POPF grade B/C. House et al. showed that the patients with retrorenal visceral fat thickness were associated with the incidence of pancreatic fistula after pancreaticoduodenectomy.[34] Moreover, fatty infiltration into the pancreatic parenchyma was demonstrated as a risk factor for POPF after pancreaticoduodenectomy;[35, 36] therefore, further studies are expected to associate VFA, fatty infiltration, and POPF.

Patients with postoperative mortality had significantly higher BMI and higher VFA than other patients, whereas neither BMI nor VFA was a risk factor for mortality.

Table 8 Univariate and multivariate analyses of risk factors for mortality

Variable	Univariate analysis		Multivariate analysis	
	P value	Odds ratio (95% CI)	*P* value	Odds ratio (95% CI)
Age, years (≥70 or <70)	.6097	0.625 (0.103–3.794)	–	–
Gender (male/female)	.3201	3.051 (0.337–27.615)	–	–
Concomitant disease (yes/no)				
Cardiovascular disease	.9796	NE	–	–
COPD	.9828	NE	–	–
Diabetes mellitus	.9690	NE	–	–
Recent smoking history[a] (yes/no)	.0329	11.036 (1.216–100.187)	.0321	28.954 (1.332–629.405)
Pulmonary function on spirogram				
%VC<80% (yes/no)	.4513	2.350 (0.254–21.716)	–	–
FEV1/FVC ratio<0.70 (yes/no)	.2844	2.688 (0.440–16.440)	–	–
Preoperative biliary drainage (yes/no)	.3719	2.274 (0.375–13.809)	–	–
Type of resection (PpPD/PD)	.9795	NE	–	–
Additional portal vein resection (yes/no)	.1101	4.407 (0.714–27.176)	.0695	14.656 (0.807–266.262)
Pancreatic texture (soft/hard)	.3185	0.326 (0.036–2.950)	–	–
Histologic diagnosis (malignant/benign)	.1191	0.238 (0.039–1.448)	.0684	0.048 (0.002–1.258)
BMI (≥25 or <25)	.0158	9.384 (1.523–57.805)	.1145	26.257 (0.453–1520.454)
VFA (≥130 or <130)	.0100	18.286 (2.005–166.773)	.7514	1.695 (0.065–44.353)
Operative time (min; ≥350 or <350)	.9763	NE	–	–
Intraoperative bleeding (ml; ≥1,000 or <1,000)	.2148	3.134 (0.515–19.053)	–	–
Red blood cell transfusion (yes/no)	.0761	7.345 (0.811–66.545)	.1175	15.023 (0.505–447.051)

CI confidence interval, *NE* not able to estimate, *COPD* chronic obstructive pulmonary disease, *%VC* percentage predicted vital capacity, *FEV1/FVC ratio* ratio of forced expiratory volume in 1 s to forced vital capacity, *PpPD* pylorus-preserving pancreaticoduodenectomy, *PD* pancreaticoduodenectomy, *BMI* body mass index, *VFA* visceral fat area

[a] Recent smoking history = smoking within 4 weeks prior to surgery[17]

Fortunately, our study had a low incidence of mortality (n= 5, 1.6%); therefore, the influence of VFA on mortality cannot be evaluated.

The limitations of our study include the facts that the racial responses to relative levels of obesity with the population in Japan differ across much of the Western countries. WHO defines obesity as BMI≥30.0, but the prevalence of the population with such a BMI is less than 3% of the general population in Japan.[37] The Western Pacific Region of the WHO has recommended lowering the BMI cutoff levels for Asian people to 25.0 for obesity[37] because of occurring of obesity-related disorders at a much lower BMI than in Caucasian populations. For this point, we defined high BMI as BMI≥25.0 kg/m^2 in this study.

In the present study, we categorized the patients into high- and low-VFA groups using the cutoff value of VFA determined to be 130 cm^2 for logistic regression analysis because VFA≥130 cm^2 has been reported to be a risk factor for cardiovascular disease,[38] metabolic syndrome,[39] and the complication of laparoscopic sigmoidectomy.[40] However, the optimal cutoff value for VFA still remains unclear, and it is essential to determine the optimal cutoff value of VFA for pancreatic surgery.

It has been demonstrated that adipose tissue is not only for fat storage but is also a metabolically active organ secreting several hormones, adipocytokines, including adiponectin, leptin, tumor necrosis factor-α, interleukin-6, angiotensinogen, and plasminogen activator inhibitor 1. Circulating adiponectin levels correlate inversely with VFA, and hypoadiponectinemia with visceral adiposity is associated with a low-grade systemic inflammatory environment.[41] Indeed, a low preoperative adiponectin level was an independent risk factor for the development of postoperative infections after colorectal cancer surgery.[42] These results suggest that adipocytokines with visceral obesity may influence postoperative complications, including PPCs.

To prevent PPCs, prophylactic respiratory physiotherapy, management of immune status, and fast-track recovery pathways including early mobilization are thought to be effective; therefore, careful perioperative management may be more essential for patients with visceral obesity.