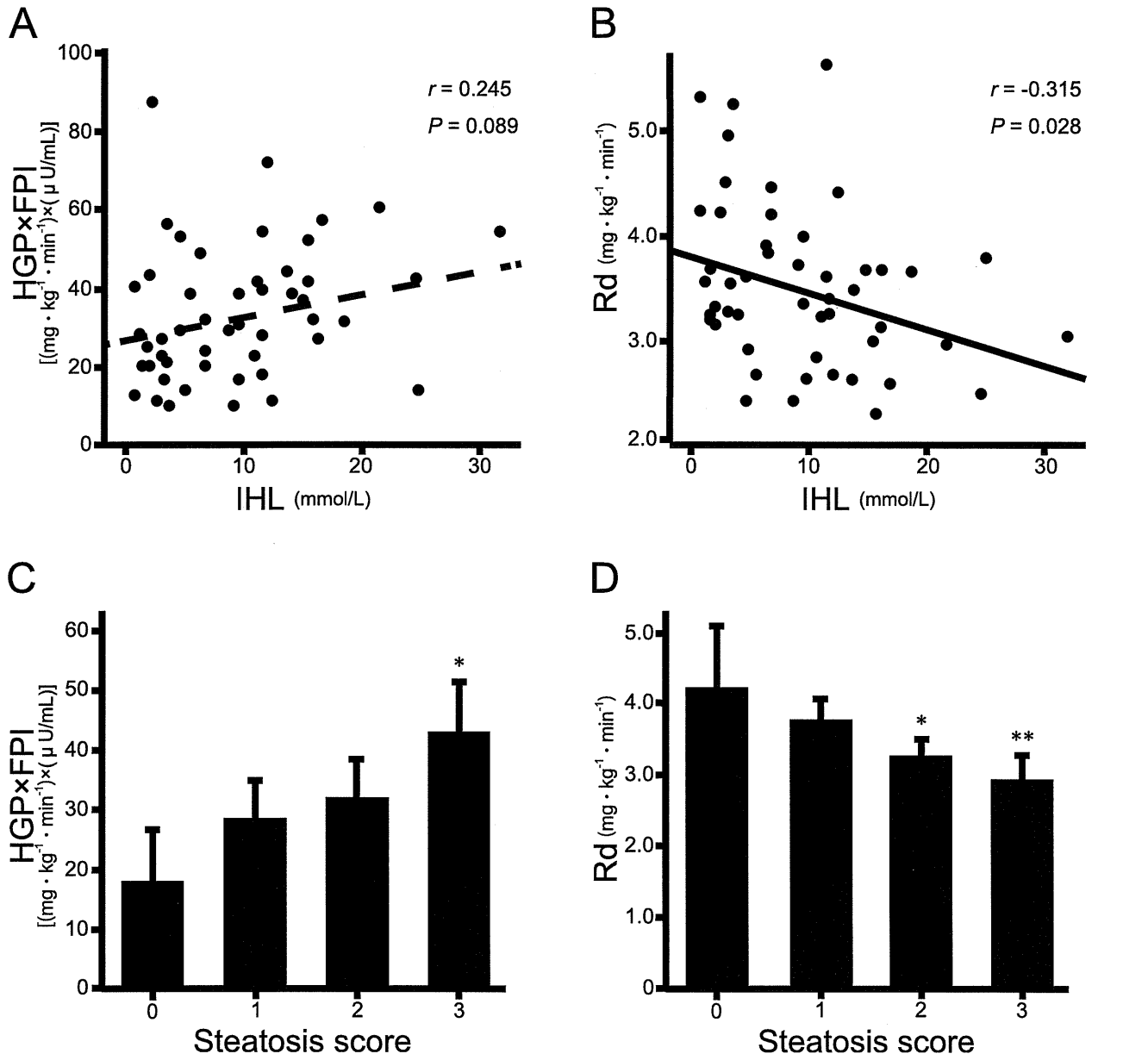

Figure 1. Correlation between liver fat and organ-specific insulin resistance (IR). (A) univariate correlation between IR in the liver (HGP×FPI) and liver fat (IHL) ($r = 0.25$, $P = 0.09$). (B) univariate correlation between skeletal muscle IR index (Rd) and liver fat (IHL) ($r = -0.32$, $P<0.05$). (C) IR in the liver (HGP×FPI) stratified by steatosis score. (D) skeletal muscle IR index (Rd) stratified by steatosis score. *$P<0.05$ vs. score 0 steatosis group. **$P<0.01$ vs. score 0 steatosis group.
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Table 3. Multiple regression models predicting HGP×FPI and Rd.

	HGP×FPI		Rd	
	β	*P*	β	*P*
Steatosis (Model 1)	0.284	0.026[a]	−0.300	0.007[b]
Steatosis (Model 2)	0.216	0.098	−0.261	0.027[a]

HGP, hepatic glucose production; FPI, fasting plasma insulin.
Model 1, adjusted for, age, sex, and body mass index; Model 2, adjusted for, age, sex, body mass index, and total fat mass.
[a]$P<0.05$,
[b]$P<0.01$.
doi:10.1371/journal.pone.0092170.t003

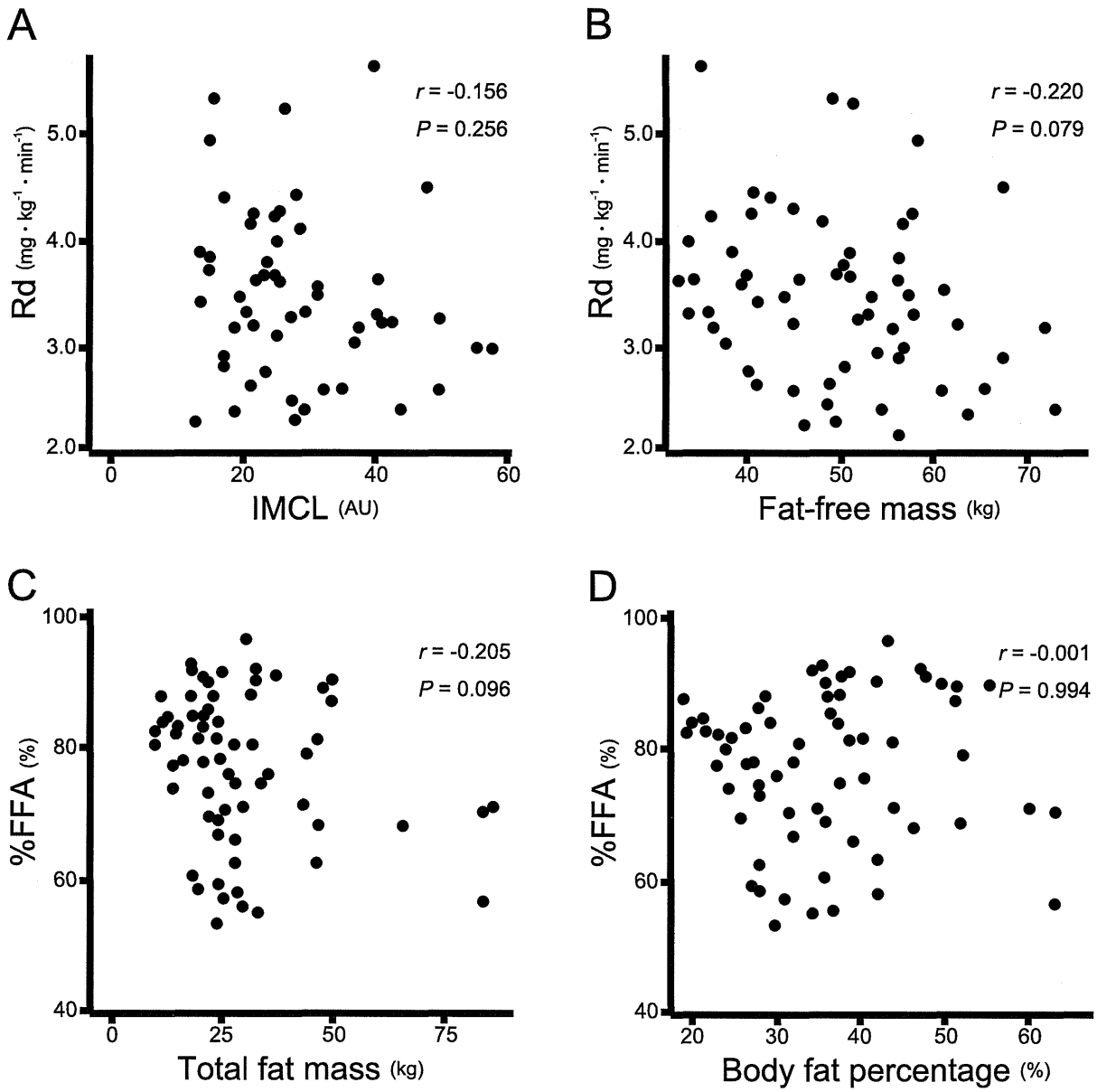

Figure 2. Correlation between ectopic fat and organ-specific insulin resistance (IR). (A) univariate correlation between skeletal muscle IR index (Rd) and intramyocellular lipid (IMCL) ($r = -0.16$, $P = 0.26$). (B) univariate correlation between Rd and fat-free mass ($r = 0.22$, $P = 0.08$). (C) univariate correlation between adipose tissue IR index (%FFA) and total fat mass ($r = -0.21$, $P = 0.10$). (D) univariate correlation between %FFA and body fat percentage ($r = -0.00$, $P = 0.99$).
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[32,33]. Lowering steady-state insulin levels by a reduced insulin infusion rate might improve the specificity of these indices to reflect organ insulin sensitivity.

In the present study, IMCL was not associated with skeletal muscle IR. The participants in this study had a wide BMI range (21.3–54.9 kg/m^2) and subjects may have different physical exercise habits with various intensities. IMCL is increased not only by obesity but also by enhanced physical fitness [34]. Therefore, absolute fat contents do not always predict IR in the skeletal muscle, thus, toxic lipids that cause IR in the skeletal muscle should be further researched. Similarly, we failed to find any relationship between fat mass or its percentage and adipose tissue IR. Although we evaluated only total fat mass, distribution of adipose tissue may potentially determine insulin action. Indeed, visceral fat, but not subcutaneous fat, is reported to be associated with %FFA [35]. Therefore, future studies should evaluate visceral and subcutaneous fat masses separately and evaluate the relation to %FFA in Japanese people.

In addition to the previously well-recognized relationship between adipose tissue mass and IR in the liver and skeletal muscle [36], the present study showed a distinct relationship between liver fat and skeletal muscle IR independently of age, sex, and BMI. Although our results are consistent with previous studies showing that liver fat plays an important role in peripheral IR as well as hepatic IR [14,15], not all associations among components of ectopic fat and organ-specific IR were examined simultaneously in these studies. Our findings suggest that hepatic steatosis *per se* is a central surrogate pathology indicative of IR in both liver and

Table 4. Univariate correlation between ectopic fat and organ-specific insulin resistance in subjects without type 2 diabetes ($n=32$).

	HGP×FPI		%HGP		Rd		Rd/SSPI		%FFA	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
HGP×FPI	1	–	−0.132	0.470	−0.551[b]	0.001	−0.554[b]	0.001	−0.095	0.607
%HGP	−0.132	0.470	1	–	0.101	0.583	0.121	0.509	0.370[a]	0.037
Rd	−0.551[b]	0.001	0.101	0.583	1	–	0.798[c]	<0.001	0.297	0.099
Rd/SSPI	−0.554[b]	0.001	0.121	0.509	0.798[c]	<0.001	1	–	0.210	0.250
%FFA	−0.095	0.607	0.370[a]	0.037	0.297	0.099	0.210	0.250	1	–
Steatosis	0.524[b]	0.002	−0.008	0.964	−0.478[b]	0.006	−0.409[a]	0.020	0.099	0.589
Grade	0.403[a]	0.022	−0.115	0.530	−0.487[b]	0.005	−0.497[b]	0.004	0.172	0.348
Stage	0.206	0.258	−0.033	0.859	−0.305	0.090	−0.194	0.286	0.114	0.534
IHL	0.198	0.403	0.155	0.515	−0.570[b]	0.009	−0.414	0.070	−0.173	0.465
IMCL	0.318	0.130	−0.334	0.110	−0.128	0.552	−0.151	0.480	−0.172	0.421
Fat-free mass	−0.017	0.926	−0.262	0.154	−0.050	0.788	−0.118	0.527	−0.321	0.078
Total fat mass	0.523[b]	0.003	0.030	0.873	−0.728[c]	<0.001	−0.586[b]	0.001	−0.082	0.660
Body fat percentage	0.488[b]	0.005	0.056	0.763	−0.729[c]	<0.001	−0.599[c]	<0.001	0.045	0.811
VO_2	−0.045	0.829	0.131	0.523	0.379	0.057	0.356	0.074	0.009	0.964

HGP, hepatic glucose production; FPI, fasting plasma insulin; SSPI, steady state plasma insulin; IHL, intrahepatic lipid; IMCL, intramyocellular lipid; VO_2, basal oxygen consumption rate per body weight.
[a]$P<0.05$,
[b]$P<0.01$,
[c]$P<0.001$.
doi:10.1371/journal.pone.0092170.t004

Table 5. Univariate correlation between ectopic fat and organ-specific insulin resistance in subjects with type 2 diabetes ($n = 37$).

	HGP×FPI		%HGP		Rd		Rd/SSPI		%FFA	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
HGP×FPI	1	–	−0.119	0.483	−0.600[c]	<0.001	−0.461[b]	0.005	−0.234	0.164
%HGP	−0.119	0.483	1	–	0.233	0.165	0.582[c]	<0.001	0.419[a]	0.010
Rd	−0.600[c]	<0.001	0.233	0.165	1	–	0.746[c]	<0.001	0.244	0.145
Rd/SSPI	−0.461[b]	0.005	0.582[c]	<0.001	0.746[c]	<0.001	1	–	0.346[a]	0.039
%FFA	−0.234	0.164	0.419[a]	0.010	0.244	0.145	0.346[a]	0.039	1	–
Steatosis	0.390[a]	0.017	−0.306	0.066	−0.459[b]	0.004	−0.486[b]	0.003	−0.340[a]	0.039
Grade	0.263	0.115	−0.023	0.894	−0.282	0.091	−0.216	0.206	−0.304	0.067
Stage	0.357[a]	0.030	−0.096	0.571	−0.478[b]	0.003	−0.473[b]	0.004	−0.168	0.320
IHL	0.286	0.133	−0.122	0.529	−0.209	0.277	−0.219	0.262	−0.197	0.306
IMCL	0.202	0.276	−0.128	0.491	−0.178	0.338	−0.220	0.243	0.025	0.893
Fat-free mass	0.084	0.626	−0.136	0.430	−0.314	0.062	−0.276	0.108	−0.508[b]	0.002
Total fat mass	0.478[b]	0.003	−0.301	0.074	−0.493[b]	0.002	−0.497[b]	0.002	−0.305	0.071
Body fat percentage	0.473[b]	0.004	−0.227	0.183	−0.362[a]	0.030	−0.374[a]	0.027	−0.049	0.775
VO_2	−0.211	0.264	0.264	0.159	0.460[a]	0.011	0.460[a]	0.012	0.212	0.261

HGP, hepatic glucose production; FPI, fasting plasma insulin; SSPI, steady state plasma insulin; IHL, intrahepatic lipid; IMCL, intramyocellular lipid; VO_2, basal oxygen consumption rate per body weight.
[a]$P<0.05$,
[b]$P<0.01$,
[c]$P<0.001$.
doi:10.1371/journal.pone.0092170.t005

skeletal muscle in patients with NAFLD. In addition, there may be a network between the liver and skeletal muscle to maintain whole body energy homeostasis. Accordingly, whether hepatic steatosis is a consequence or cause of skeletal muscle IR remains uncertain because a longitudinal observation of the relationship is lacking. One hypothesis is that skeletal muscle IR causes obesity and subsequent hepatic steatosis as experimentally shown in mice with muscle-selective IR [37]. Indeed, Flannery et al. recently reported that skeletal muscle IR promotes increased hepatic *de novo* lipogenesis and hepatic steatosis in the elderly [38]. A second hypothesis is the neuronal pathway from the liver might modulate peripheral insulin sensitivity [11]. A third hypothesis is that some nutrients, such as fatty acids and amino acids, might link hepatic steatosis and skeletal muscle IR [39]. A fourth hypothesis is that a liver-derived hormone (a hepatokine) affects the distant organ insulin sensitivity. We previously isolated hepatokine selenoprotein P, which is overproduced under an overnutrition state and causes IR both in the liver and skeletal muscle [13]. In addition, serum levels of selenoprotein P are inversely associated with serum levels of adiponectin [40] that enhance skeletal muscle insulin sensitivity [12]. Therefore, overproduction of selenoprotein P in association with hepatic steatosis, by directly or indirectly lowering adiponectin levels, causes skeletal muscle IR.

There are several limitations to this study. First, this was an observational study, and we were unable to examine causal associations. A large-scale longitudinal study is needed to clarify whether hepatic steatosis is a consequence or cause of skeletal muscle IR. Second, many of the study subjects had glucose intolerance/diabetes, although the severity was relatively mild as shown by the OGTT. Therefore, IR of each organ was possibly greater in our study subjects than in the general population, which could have influenced the results. Third, fifteen out of 69 subjects were taking metformin which might influence hepatic glucose production. However, major study results were similar in diabetic subjects, non-diabetic subjects, and subjects without metformin (data not shown). Fourth, we did not collect arterial or arterialized blood samples to perform the insulin clamp because these were not included in the manufacturer's protocol of the artificial pancreas model STG-55. Further study should be required to confirm our conclusion by using arterial or arterialized blood samples.

In summary, the present study revealed an unexpected lack of an association between fat and local organ-specific IR in the skeletal muscle and adipose tissue. Instead, liver fat is strongly associated with skeletal muscle IR as well as with liver IR, suggesting a central role of fatty liver in the development of IR and that a network exists between liver and skeletal muscle to maintain whole-body energy homeostasis.

Supporting Information

Figure S1 Correlation between ectopic fat and insulin resistance (IR) in the liver, skeletal muscle, and adipose tissue. Liver fat (steatosis score) was associated with skeletal muscle IR index (Rd) as well as with IR in the liver (HGP×FPI). Intramyocellular lipid was not associated with skeletal muscle IR index (Rd). Total fat mass was associated with HGP×FPI and Rd, but not with adipose tissue IR index (%FFA).
(PDF)

Table S1 Multiple regression models predicting HGP×FPI and Rd in subjects without type 2 diabetes ($n = 32$). HGP, hepatic glucose production; FPI, fasting plasma insulin Model 1, adjusted for, age, sex, and body mass index; Model 2, adjusted for, age, sex, body mass index, and total fat mass.
(DOC)

Table S2 Multiple regression models predicting HGP×FPI and Rd in subjects with type 2 diabetes ($n = 37$). HGP, hepatic glucose production; FPI, fasting plasma insulin Model 1, adjusted for, age, sex, and body mass index; Model 2, adjusted for, age, sex, body mass index, and total fat mass.
(DOC)

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Author Contributions

Conceived and designed the experiments: TT. Performed the experiments: KK TT YT HM TO. Analyzed the data: KK TT. Contributed reagents/materials/analysis tools: YR KT. Wrote the paper: KK TT. Contributed to discussion and reviewed the manuscript: SN MM OM SK.

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ORIGINAL ARTICLE

P53, hTERT, WT-1, and VEGFR2 are the most suitable targets for cancer vaccine therapy in HLA-A24 positive pancreatic adenocarcinoma

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Abstract Cancer vaccine therapy is one of the most attractive therapies as a new treatment procedure for pancreatic adenocarcinoma. Recent technical advances have enabled the identification of cytotoxic T lymphocyte (CTL) epitopes in various tumor-associated antigens (TAAs). However, little is known about which TAA and its epitope are the most immunogenic and useful for a cancer vaccine for pancreatic adenocarcinoma. We examined the expression of 17 kinds of TAA in 9 pancreatic cancer cell lines and 12 pancreatic cancer tissues. CTL responses to 23 epitopes derived from these TAAs were analyzed using enzyme-linked immunospot (ELISPOT), CTL, and tetramer assays in 41 patients, and factors affecting the immune responses were investigated. All TAAs were frequently expressed in pancreatic adenocarcinoma cells, except for adenocarcinoma antigens recognized by T cells 1, melanoma-associated antigen (MAGE)-A1, and MAGE-A3. Among the epitopes recognized by CTLs in more than two patients in the ELISPOT assay, 6 epitopes derived from 5 TAAs, namely, MAGE-A3, p53, human telomerase reverse transcriptase (hTERT), Wilms tumor (WT)-1, and vascular endothelial growth factor receptor (VEGFR)2, could induce specific CTLs that showed cytotoxicity against pancreatic cancer cell lines. The frequency of lymphocyte subsets correlated well with TAA-specific immune response. Overall survival was significantly longer in patients with TAA-specific CTL responses than in those without. P53, hTERT, WT-1, and VEGFR2 were shown to be attractive targets for immunotherapy in patients with pancreatic adenocarcinoma, and the induction of TAA-specific CTLs may improve the prognosis of these patients.

Keywords Epitope · Immunotherapy · Cytotoxic T lymphocyte (CTL) · Enzyme-linked immunospot (ELISPOT)

Abbreviations

CTL	Cytotoxic T lymphocyte
TAA	Tumor-associated antigen
ELISPOT	Enzyme-linked immunospot
MAGE	Melanoma-associated antigen
hTERT	Human telomerase reverse transcriptase
WT-1	Wilms tumor-1
VEGFR	Vascular endothelial growth factor receptor
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction

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Introduction

Pancreatic adenocarcinoma is the fourth leading cause of cancer death worldwide [1]. Despite recent advances in diagnostic techniques, pancreatic adenocarcinoma is diagnosed at an advanced stage in most patients and, consequently, the overall 5-year survival rate is <5 % [2]. Thus, the development of a new treatment option is needed to improve the prognosis of pancreatic cancer patients without toxicity.

Immunotherapy is one of the most attractive therapies as a new treatment procedure for melanoma and other solid tumors [3]. Recent technical advances have enabled the identification of various tumor-associated antigens (TAAs) [4–21]; however, few of their epitopes are inducers of cytotoxic T lymphocyte (CTL) responses against tumors [22]. Several kinds of epitope have also been identified in patients with pancreatic adenocarcinoma [23, 24]. However, previous studies focused on the identification and evaluation of a particular antigen, and different TAAs have not yet been compared simultaneously; therefore, little is known about which epitope is the most immunogenic and useful in eliciting clinical responses in pancreatic adenocarcinoma patients.

In the present study, we compared CTL responses with various TAA-derived epitopes in identical patients with pancreatic adenocarcinomas and examined the factors that affect immune responses. This approach provided information that is useful for selecting immunogenic TAAs and suitable patients and developing a new immunotherapy for pancreatic adenocarcinoma.

Materials and methods

Patients and clinical information

In this study, we examined 41 HLA-A24-positive patients with pancreatic adenocarcinoma and 14 healthy volunteers who were HLA-A24-positive, but did not have any cancers, as negative controls. Fine-needle biopsy, a surgical specimen, or autopsy was used for the pathological diagnosis of pancreatic adenocarcinoma in 18 patients. Diagnosis of the remaining 23 patients was achieved using the radiological findings of computed tomography and/or magnetic resonance imaging. We investigated patient background, treatment procedures, and outcomes.

Clinical information was obtained from the medical records of patients. We evaluated the tumor stage using TNM staging of the Union Internationale Contre Le Cancer (UICC) system (7th version) (UICC stage). The frequency of lymphocyte subsets was calculated by dividing the absolute lymphocyte count by the absolute leukocyte count. HLA typing of peripheral blood mononuclear cells (PBMCs) from patients and healthy volunteers was performed by the reverse sequence-specific oligonucleotide with polymerase chain reaction (PCR-RSSO). This study was approved by the Ethics Committees of Kanazawa University (No. 1237) and Kanazawa Medical Center (No. 17), and all patients gave written informed consent to participate in accordance with the Helsinki Declaration.

Table 1 Peptides used in this study

Peptide No.	TAA	Amino acid sequence	Reference
1	$ART1_{188}$	EYCLKFTKL	[14]
2	$ART4_{161}$	AFLRHAAL	[11]
3	$ART4_{899}$	DYPSLSATDI	[11]
4	$Cyp\text{-}B_{109}$	KFHRVIKDF	[7]
5	$Cyp\text{-}B_{315}$	DFMIQGGDF	[7]
6	Lck_{208}	HYTNASDGL	[8]
7	Lck_{488}	DYLRSVLEDF	[8]
8	$MAGE\text{-}A1_{135}$	NYKHCFPEI	[6]
9	$MAGE\text{-}A3_{195}$	IMPKAGLLI	[16]
10	$SART1_{690}$	EYRGFTQDF	[12]
11	$SART2_{899}$	SYTRLFLIL	[13]
12	$SART3_{109}$	VYDYNCHVDL	[21]
13	$Her\text{-}2/neu_{8}$	RWGLLLALL	[17]
14	$p53_{161}$	AIYKQSQHM	[18]
15	$p53_{204}$	EYLDDRNTF	[5]
16	$MRP3_{765}$	VYSDADIFL	[20]
17	$MRP3_{503}$	LYAWEPSFL	[20]
18	$hTERT_{461}$	VYGFVRACL	[4]
19	$hTERT_{324}$	VYAETKHFL	[4]
20	$WT\text{-}1_{235}$	CMTWNQMNL	[15]
21	$VEGFR2_{169}$	RFVPDGNRI	[19]
22	$VEGFR1_{1084}$	SYGVLLWEI	[10]
23	$survivin2B_{80}$	AYACNTSTL	[9]
24	HIV env_{584}	RYLRDQQLL	[25]
25	CMV $pp65_{328}$	QYDPVAALF	[26]

Synthetic peptides and preparation of PBMCs

The 23 epitopes derived from 17 different TAAs used in the present study are listed in Table 1. We selected epitopes that had previously been identified as HLA-A24-restricted and suggested to have immunogenicity in various cancers not restricted to pancreatic cancer [4–21]. Epitopes derived from the HIV envelope protein (HIV env_{584}) [25] and cytomegalovirus (CMV) pp65 (CMV$pp65_{328}$) [26] were also used to assess T cell responses. Peptides were synthesized at Mimotope (Melbourne, Australia), Sumitomo Pharmaceuticals (Osaka, Japan), COSMO BIO Co. (Tokyo, Japan), and Scrum Inc. (Tokyo, Japan). Purities were determined to be >80 % by analytical high-performance

liquid chromatography (HPLC). PBMCs were separated as described below; heparinized venous blood was diluted in phosphate-buffered saline (PBS) and loaded on Ficoll-Histopaque (Sigma, St. Louis, MO) in 50-ml tubes. After centrifugation at 2,000 rpm for 20 min at room temperature, PBMCs were harvested from the interphase, resuspended in PBS, centrifuged at 1,400 rpm for 10 min, and finally resuspended in complete culture medium consisting of RPMI (GibcoBRL, Grand Island, NY), 10 % heat-inactivated FCS (Gibco BRL), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL).

Cell lines

The HLA-A*2402 gene-transfected C1R cell line (C1R-A24) was cultured in RPMI 1640 medium containing 10 % FCS and 500 μg/ml hygromycin B (Sigma, St. Louis, MO), and K562 was cultured in RPMI 1640 medium containing 10 % FCS [27]. MiaPaca2, AsPC1, BxPC3, Panc-1, CAPAN1, and CAPAN2 were purchased from the American Type Culture Collection (VA, USA). YPK-1 and YPK-2 were kind gifts from Prof. Oka and Dr. Yoshimura (Yamaguchi University Graduate School of Medicine, Yamaguchi, Japan). PK-1 was provided by the RIKEN BRC through the National Bio-Resource Project of MEXT, Japan. Human pancreatic cancer cell lines were cultured in DMEM (GibcoBRL) or RPMI 1640 medium containing 10 % fetal calf serum (FCS). All media contained 100 U/mL penicillin and 100 μg/mL streptomycin.

RNA preparation and real-time PCR

The expression of TAA messenger RNA (mRNA) in human pancreatic cancer cell lines and pancreatic adenocarcinoma tissues was analyzed by real-time polymerase chain reaction (PCR). Cell lines were harvested, centrifuged, and washed with PBS, and total RNA was then isolated using Quick-Gene (Fuji Film, Tokyo). Total RNA from frozen pancreatic adenocarcinoma samples was isolated using a GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) according to the manufacturer's protocol. cDNA was synthesized from 150 ng of total RNA using a high-capacity cDNA reverse transcription kit (PE Applied Biosystems, CA, USA) and was then mixed with TaqMan Universal Master Mix (PE Applied Biosystems) and each TaqMan probe. Primer pairs and probes for various TAAs and β-actin were obtained from the TaqMan assay reagents library. Thermal cycling conditions were 25 °C for 10 min, 37 °C for 120 min, and 85 °C for 1 min. cDNA was subjected to quantitative real-time PCR analyses targeting various TAAs and β-actin. Analyses were performed using the StepOne Real-Time PCR system and StepOne v2.0 software. Relative gene expression values were determined. Data are presented as fold differences in TAA expression normalized to the housekeeping gene β-actin as an endogenous reference.

Enzyme-linked immunospot assay (ELISPOT assay)

Ninety-six-well plates (Millititer, Millipore, Bedford, MA) were coated with anti-human interferon-γ (IFN-γ) (Mabtech, Nacka, Sweden) at 4 °C overnight and then washed 4 times with sterile PBS. The plates were then blocked with RPMI 1640 medium containing 5 % FCS for 2 h at room temperature. A total of 300,000 unfractionated PBMCs were added in duplicate cultures of RPMI 1640 containing 5 % FCS together with the peptides at 10 μg/ml. After 24 h, the plates were washed 8 times with PBS and incubated overnight with 100 μl of the biotin-conjugated anti-human IFN-γ antibody. After another 4 washes with PBS, streptavidin-AP was added for 2 h. Finally, the plates were washed again 4 times with PBS and developed with freshly prepared NBT/BCIP solution (Biorad, Hercules, CA). The reaction was stopped by washing with distilled water and drying at room temperature. Colored spots with fuzzy borders, which indicated the presence of IFN-γ-secreting cells, were counted. The number of specific spots was determined by subtracting the number of spots in the absence of the antigen. Responses were considered positive if 10 or more specific spots were detected and if the number of spots in the presence of an antigen was at least two-fold than that in its absence.

Peptide-specific CTL induction and cytotoxicity assay

Synthetic peptide-specific T cells were expanded from PBMCs in 96-well round-bottom plates (NUNC, Naperville, IL). Four hundred thousand cells/well were stimulated with synthetic peptides at 10 μg/ml, 10 ng/ml rIL-7, and 100 pg/ml rIL-12 (Sigma) in RPMI 1640 supplemented with 10 % heat-inactivated human AB serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cultures were restimulated with 10 μg/ml peptide, 20 U/ml rIL-2 (Sigma), and 10^5 mitomycin C-treated autologous PBMCs as feeder cells on days 7 and 14. One hundred microliters of RPMI medium with 10 % human Ab serum and rIL-2 at a final concentration of 10 U/ml were added to each well on days 4, 11, and 18. The cytotoxicity assay was conducted on day 22.

The C1R-A24 and human pancreatic cancer cell lines were used as target cells for the ^{51}Cr release assay. C1R-A24 cells were incubated overnight with 10 μg/ml synthetic peptides and labeled with 25 μCi of ^{51}Cr for 1 h. Pancreatic cancer cell lines were also labeled with 25 μCi of ^{51}Cr for 1 h without incubation with peptides. After three washes with PBS, target cells were plated at 3,000 cells/well in complete medium

in round-bottom 96-well plates. Unlabeled K562 (120,000 cells/well) was added to reduce non-specific lysis. Peptide-stimulated PBMCs were added at various effector-to-target ratios as indicated. Maximum release was determined by the lysis of ^{51}Cr-labeled targets with 5 % Triton X-100 (Sigma Chemical). Spontaneous release was <10 % of maximum release for all experiments, except for when it was <15 % when the target cells were human pancreatic cancer cell lines. Percent-specific cytotoxicity was determined using the following formula: 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release), and specific cytotoxic activity was calculated as follows: (cytotoxic activity in the presence of the peptide) − (cytotoxic activity in the absence of the peptide). Specific cytotoxicity of more than 10 % was considered to be positive.

Tetramer staining and flow cytometry

TAA-specific tetramers were purchased from Medical Biological Laboratories Co., Ltd. (Nagoya, Japan). Tetramer staining was performed as described below. One million isolated PBMCs or peptide-specific CTLs pulsed with TAA-derived peptides were washed, resuspended in 200 μl of PBS without calcium or phosphate, and stained with 40 μg/ml tetrameric complexes and monoclonal antibodies against cell surface proteins for 30 min at room temperature. The following monoclonal antibodies were used: anti-CD8-APC (BD PharMingen, San Diego, CA), anti-CCR7-FITC, anti-CD45RA-PerCP, and tetramer-PE. Cells were washed, fixed with 0.5 % paraformaldehyde/PBS, and analyzed on a Becton–Dickinson FACSAria II system.

Statistical analysis

Fisher's exact test and unpaired Student's *t* test were used to analyze the effect of variables on immune responses in pancreatic cancer patients. Overall survival was calculated from the day of pancreatic cancer diagnosis until the date of death or the last day of the follow-up period. Cumulative survival proportions were calculated using the Kaplan–Meier method, and any differences were evaluated using the log-rank test. A p value of <0.05 was considered to be significant, and all the tests were two-sided. All statistical analyses were performed using the SPSS statistical software program package (SPSS version 11.0 for Windows).

Results

Patients

Patient characteristics are summarized in the Supplementary Table. The median age of patients was 72 years, and patients included 24 males (59 %). The main localization of the tumors was the pancreatic head in 39 % of patients and the pancreatic body or tail in 61 %. The majority of patients (93 %) had advanced-stage cancer, namely, UICC stage III or IV. Therapeutic procedures mainly involved chemotherapy consisting of protocols such as gemcitabine monotherapy, S-1 monotherapy, or a combination of both drugs. Only 11 patients received the best supportive therapy to relieve physical and spiritual pain. A total of 61 % of patients had died by the last day of the follow-up period, and the median overall survival time of patients was 7.2 months.

TAA expression in pancreatic cancer cell lines and human cancer tissues

We evaluated the expression of 17 different TAAs in 9 human pancreatic cancer cell lines using real-time PCR. Although differences were observed from cell to cell, TAAs were expressed in more than 40 % of pancreatic adenocarcinoma cell lines, except for adenocarcinoma antigens recognized by T cells (ART)1 (11 %) and ART4 (33 %) (Table 2). We then investigated TAA expression in 7 surgical and 5 autopsy specimens. The expression of most TAAs in pancreatic adenocarcinoma specimens was similar to or more frequent than that in human pancreatic cancer cell lines, except for melanoma-associated antigen (MAGE)-A1 and MAGE-A3 (Table 2).

Detection of TAA-specific T cells by IFN-γ ELISPOT analysis

IFN-γ ELISPOT responses were evaluated with PBMCs to determine how frequently T cells respond to TAA-derived peptides and control peptides in patients with pancreatic adenocarcinoma (Fig. 1a). Positive responses to at least one TAA-derived peptide were observed in 28 of 41 (68 %) patients. On the other hand, 14 of 23 (61 %) peptides were recognized by T cells obtained from at least one patient. $ART1_{188}$, $ART4_{161}$, $ART4_{899}$, lymphocyte-specific protein tyrosine kinase $(Lck)_{208}$, $MAGE\text{-}A3_{195}$, $p53_{161}$, human telomerase reverse transcriptase $(hTERT)_{461}$, $hTERT_{324}$, Wilms tumor $(WT)\text{-}1_{235}$, vascular endothelial growth factor receptor $(VEGFR)2_{169}$, and $VEGFR1_{1084}$ were recognized in more than two patients, which suggested that these peptides have the potential to be immunogenic. Peptides 24 ($HIVenv_{584}$) and 25 ($CMVpp65_{328}$) were recognized in 0 and 38 % of patients, respectively.

Peptides $ART4_{161}$, $ART4_{899}$, Cyclophilin B $(Cyp\text{-}B)_{315}$, Lck_{208}, $hTERT_{324}$, and $VEGFR1_{1084}$ were recognized in more than one healthy volunteer, and/or the percentage of positive responses was higher in healthy volunteers than in pancreatic adenocarcinoma patients, which indicated

Table 2 Expression of various TAAs mRNA in pancreatic cancer cell lines and pancreatic cancer tissues measured by real-time PCR

TAA	Primer	Positive cell lines/ cell lines tested *n* (%)	Positive specimens/ specimens tested *n* (%)
ART1	Hs00188841_m1	1/9 (11)	5/12 (42)
ART4	Hs00221465_m1	3/9 (33)	11/12 (92)
CypB	Hs00168719_m1	9/9 (100)	12/12 (100)
Lck	Hs00178427_m1	8/9 (89)	11/12 (92)
MAGEA1	Hs00607097_m1	4/9 (43)	1/12 (8)
MAGEA3	Hs00366532_m1	4/9 (43)	1/12 (8)
SART1	Hs00193002_m1	9/9 (100)	12/12 (100)
SART2	Hs00203441_m1	9/9 (100)	12/12 (100)
SART3	Hs00206829_m1	9/9 (100)	12/12 (100)
HER2/neu	Hs00170433_m1	9/9 (100)	12/12 (100)
p53	Hs00153340_m1	9/9 (100)	12/12 (100)
MRP3	Hs00358656_m1	9/9 (100)	12/12 (100)
hTERT	Hs00162669_m1	9/9 (100)	9/12 (75)
WT-1	Hs00240913_m1	5/9 (56)	9/12 (75)
VEGFR2	Hs00911700_m1	5/9 (56)	11/12 (92)
VEGFR1	Hs01052961_m1	6/9 (67)	12/12 (100)
Survivin	Hs00153353_m1	9/9 (100)	12/12 (100)

that the responses to these peptides were not specific to T cells from patients with pancreatic adenocarcinoma (Fig. 1b). In other words, peptides $ART1_{188}$, MAGE-$A3_{195}$, $p53_{161}$, $hTERT_{461}$, WT-1_{235}, and $VEGFR2_{169}$ have specific immunogenic potential in patients with pancreatic adenocarcinoma.

The number of peptide-specific IFN-γ-producing T cells was counted to examine the frequency of T cells responsive to TAA-derived peptides. A range of 10–46 T cells per 300,000 PBMCs in patients with pancreatic adenocarcinoma produced IFN-γ (Fig. 1c).

TAA-specific CTL induction and cytotoxic activity

We attempted to induce peptides specific to CTLs from the PBMCs of pancreatic adenocarcinoma patients. Cytotoxicity assays were performed in more than five patients for each peptide. Of the 11 peptides recognized in more than two patients in the IFN-γ ELISPOT assay, 6 peptides (MAGE-$A3_{195}$, $p53_{161}$, $hTERT_{461}$, $hTERT_{324}$, WT-1_{235}, and $VEGFR2_{169}$) could induce their specific CTLs, which were confirmed to be able to respond to C1RA24 cells pulsed with corresponding peptides by the cytotoxicity assay, as shown in Fig. 2a.

We conducted a cytotoxicity assay to determine whether peptide-specific CTLs from healthy volunteers could show their cytotoxic activity against pancreatic carcinoma cell lines. $P53_{161}$-, $hTERT_{461}$-, and $hTERT_{324}$-specific CTLs showed cytotoxicity against YPK-2 (HLA-A24-, p53-, and hTERT-positive), but not against Panc-1 (HLA-A24-negative, p53- and hTERT-positive). MAGE-$A3_{195}$-, WT-1_{235}-, and $VEGFR2_{169}$-specific CTLs also showed cytotoxic activity against YPK-2 (HLA-A24-, MAGE-A3-, WT-1-, and VEGFR2-positive), but not against YPK-1 (HLA-A24-positive, MAGE-A3-, WT-1-, and VEGFR2-negative). Representative data are shown in Fig. 2b.

Phenotypic analysis of TAA-derived peptides specific to T cells

To analyze the characteristics of TAA-derived peptides specific to T cells and select the appropriate epitope for immunotherapy in patients with pancreatic adenocarcinoma, we performed phenotypic analysis by tetramer staining and FACS analysis. We first attempted to detect MAGE-$A3_{195}$-, $hTERT_{461}$-, and WT-1_{235}-specific tetramer-positive T cells in PBMCs and CTLs induced by the corresponding peptides in healthy volunteers. The ratio of tetramer-positive T cells was increased in CTLs and their frequencies were 1.481–2.930 % of $CD8^+$ T cells, suggesting that these tetramers work well (Fig. 3a). We also conducted similar assays in pancreatic adenocarcinoma patients and detected tetramer-positive T cells in CTLs (Fig. 3b).

We then examined the naïve/effector/memory phenotype of tetramer-positive cells in the PBMCs of patients. The memory phenotype was investigated by the criterion of CD45RA/CCR7 expression [28]. In tetramer analysis, the frequencies of MAGE-$A3_{195}$-, $hTERT_{461}$-, and WT-1_{235}-specific tetramer-positive T cells were 0.003–0.044,

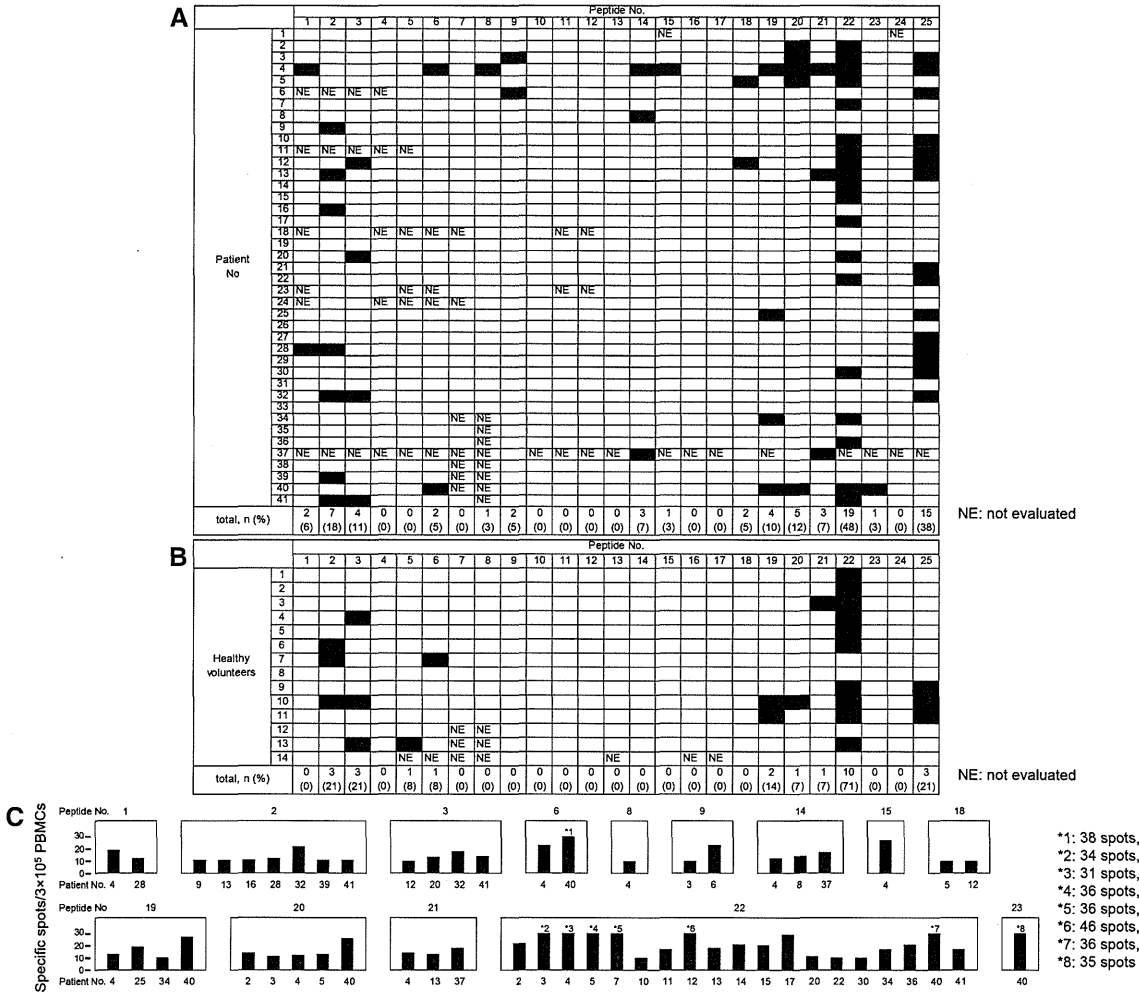

Fig. 1 T cell responses to TAA-derived peptides and control peptides in pancreatic adenocarcinoma patients **a** and healthy volunteers **b**. T cell responses were evaluated by the IFN-γ ELISPOT assay. Responses were considered positive if 10 or more specific spots were detected and if the number of spots in the presence of an antigen was at least twofold that in its absence. *Black boxes* indicate positive responses. **c** The frequency of TAA-specific IFN-γ-producing T cells evaluated by the ELISPOT assay. *Black bars* indicate the response of one patient

0.006–0.053, and 0.030–0.191 % of $CD8^+$ T cells, respectively. The frequency of $CD45RA^-/CCR7^+$ (central memory), $CD45RA^-/CCR7^-$ (effector memory), and $CD45RA^+/CCR7^-$ (effector) T cells in tetramer-positive cells depended on the patient and all phenotypes were observed in all patients, except for patients 1, 8, 28, 29, and 4 (Supplementary Fig. 1).

TAA-specific T cell responses and clinical features of pancreatic cancer patients

In the present study, we analyzed the clinical features that can affect TAA-specific immune responses. When we divided patients into two groups based on their frequencies of lymphocyte subsets in peripheral leukocytes (<24 %, the median value among all patients, or equal to or more than 24 %) and the strength of TAA-specific immune responses into three groups according to the frequency of TAA-specific T cells (<10 specific spots on ELISPOT assays, no response; 10–19 specific spots, weak response; equal to or more than 20 specific spots, strong response), the patients with more lymphocyte subsets in peripheral leukocytes showed stronger TAA-specific T cell responses (Supplementary Fig. 2). On the other hand, we could not find any relationship between TAA-specific immune responses and other clinical characteristics such as age, sex, tumor marker levels, UICC stage, or metastasis status.

We also analyzed the correlation between T cell responses and the prognosis of pancreatic cancer patients. The median overall survival time of patients with T cell responses to at least one TAA-derived peptide evaluated

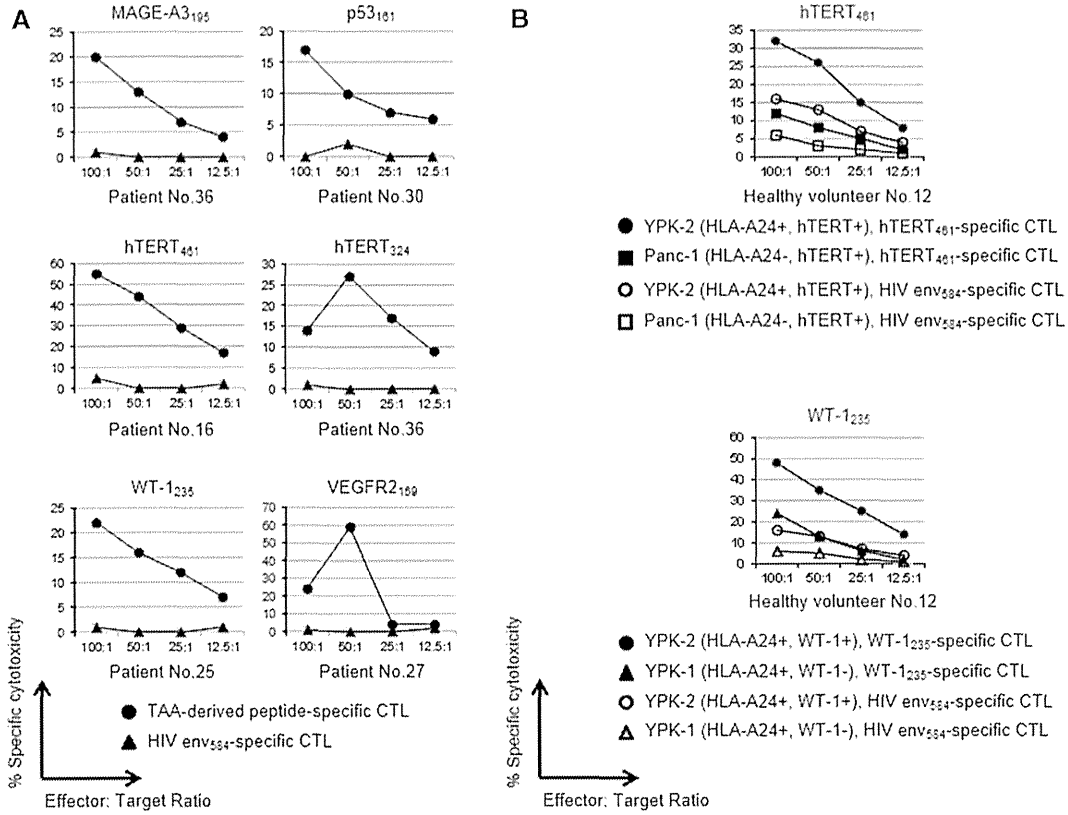

Fig. 2 **a** T cell responses to peptides evaluated by the cytotoxicity assay. Peptide-specific CTL induction and cytotoxicity assays were performed on the PBMCs from at least five patients, and representative data are shown when peptide-specific CTLs were induced in one or more patients. A percent-specific cytotoxicity of more than 10 % was considered to be positive. Six peptides: 9, 14, 18, 19, 20, and 21, could induce their specific CTLs, and these could respond to CIRA24 cells pulsed with the corresponding peptides in the cytotoxicity assay. **b** Cytotoxic activity against the pancreatic carcinoma cell lines of TAA-specific CTLs from healthy volunteers evaluated by the cytotoxicity assay. Cytotoxicity was stronger against pancreatic carcinoma cells that were HLA-A24-restricted and expressed corresponding TAAs than against those not HLA-A24-restricted or not expressing corresponding TAAs

by the ELISPOT assay was 12.2 months, which was significantly longer than that without T cell responses (4.3 months) ($p = 0.013$) (Fig. 4a). On the other hand, no correlation was observed between positive T cell responses and CMV-derived peptides and clinical outcomes (Fig. 4b), suggesting that TAA-specific T cell responses, but not the general immune response, is a prognostic factor in patients with pancreatic adenocarcinoma. The frequencies of regulatory T cells or the ratio of regulatory T cells to $CD8^+$ T cells had no impact on the outcomes of patients in this study.

Discussion

Immunotherapy is considered to be a fourth treatment procedure for cancer following surgical resection, radiotherapy, and chemotherapy [29]. Cancer vaccine therapy was previously shown to convey survival benefits to prostate cancer patients in a clinical phase III trial [30], and some candidates of other cancers have been identified and separately evaluated to determine whether a CTL response can be elicited, with the subsequent elimination of cancer cells and improvement in outcomes. Although a successful clinical response depends on how much tumor antigens elicit their specific CTLs, which are the most important effector cells for antitumor immune responses, to the best of our knowledge, no studies have attempted to identify which epitopes are optimal for peptide vaccine therapy in patients with pancreatic adenocarcinoma. Therefore, we simultaneously compared peptide-specific T cell responses among various TAAs in 41 identical patients with pancreatic adenocarcinoma under the same experimental conditions.

Therapeutic function is the most important factor to consider when determining the usefulness of cancer antigens for peptide vaccine therapy. However, it is very difficult to compare the efficacy of more than one epitope, especially in patients with pancreatic adenocarcinoma whose survival time is very short. Under such circumstances, immunogenicity, specificity, oncogenicity, expression levels, % of

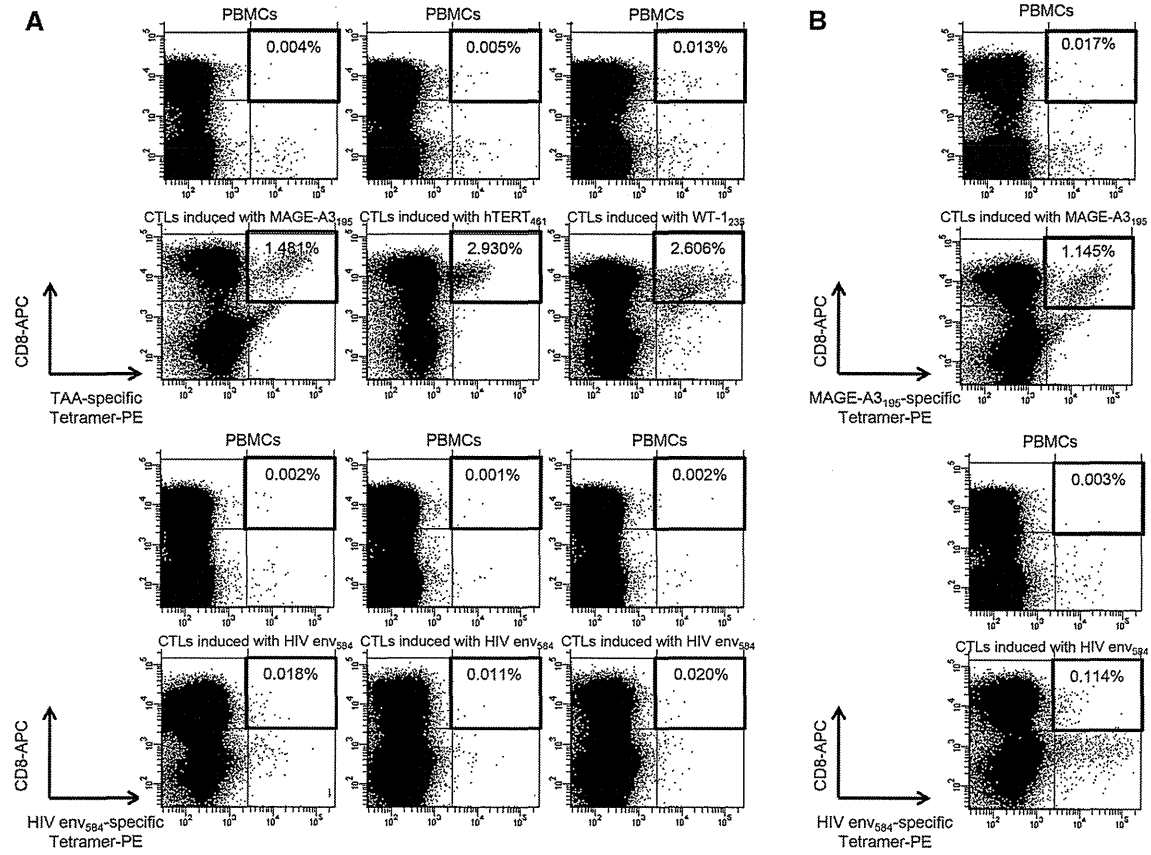

Fig. 3 Detection of TAA-specific, HLA-A24-tetramer$^+$, and CD8$^+$ lymphocytes in PBMCs from healthy volunteers and pancreatic adenocarcinoma patients. **a** Tetramer analyses were performed on eight healthy volunteers for each peptide (MAGE-A3$_{195}$, hTERT$_{461}$, and WT-1$_{235}$). Tetramer$^+$ and CD8$^+$ T cells were detectable in both PBMCs and CTLs induced by their corresponding peptides in at least one healthy volunteer, and representative data are shown in cases in which the ratio of tetramer$^+$ and CD8$^+$ T cells to CD8$^+$ T cells was higher in CTLs induced with each TAA-derived peptide than in PBMCs. **b** Tetramer analyses were performed on pancreatic adenocarcinoma patients using PBMCs and CTLs, which were induced with TAA-derived peptides and showed cytotoxicity against pancreatic cancer cell lines in cytotoxicity assay. Levels of tetramer$^+$ and CD8$^+$ T cells were higher in CTLs induced with TAA-derived peptides than in PBMCs. Representative data are shown in cases in which the ratio of tetramer$^+$ and CD8$^+$ T cells to CD8$^+$ was 0.017 % in PBMCs and 1.145 % in MAGE-A3$_{195}$-specific CTLs

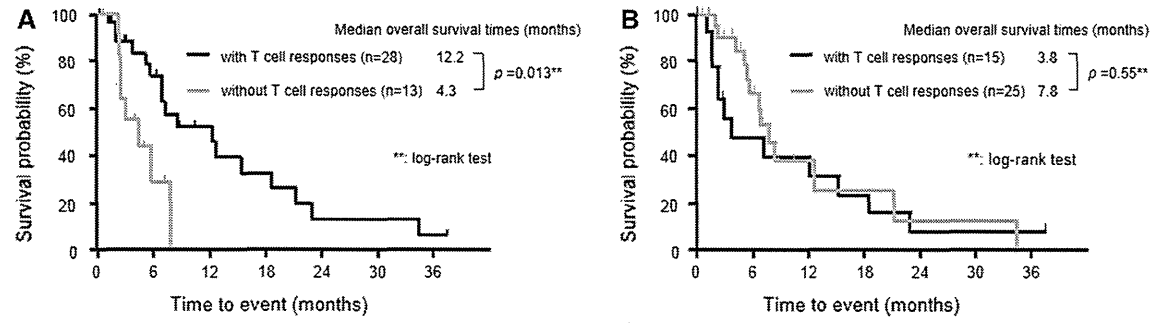

Fig. 4 Kaplan-Meier plot of the overall survival of pancreatic cancer patients according to **a** TAA-specific T cell responses and **b** T cell responses to CMV-derived peptides. **a** TAA-specific T cell responses were defined as positive if 10 or more specific spots to at least one TAA-derived peptide were detected on the ELISPOT assay. The overall survival time of patients with TAA-specific T cell responses was significantly longer than that of patients without TAA-specific T cell responses. **b** T cell responses to CMV-derived peptides were defined as positive if 10 or more specific spots to CMV-derived peptides were detected on ELISPOT assays. No correlation was observed between positive T cell responses to CMV-derived peptides and the clinical outcomes of patients

positive cells, and the number of patients with antigen-positive cancer are considered to be alternative criteria [31]. On the basis of our results, $p53_{161}$, $hTERT_{461}$, $WT\text{-}1_{235}$, and $VEGFR2_{169}$ are considered the most optimal epitopes that satisfy all of the above criteria for peptide vaccine therapy in pancreatic adenocarcinoma patients. Although $MAGE\text{-}A3_{195}$ showed immunogenicity, its expression did not appear to be high in pancreatic adenocarcinoma tissue [32]. Therefore, it may be a candidate for cancer vaccine therapy when MAGE-A3 is confirmed to be overexpressed in pancreatic cancer tissue.

A mutation in the p53 gene and overexpression of the p53 protein have been reported previously in pancreatic adenocarcinoma [33], and all pancreatic cancer cell lines and specimens used in our study expressed p53. Some strategies targeting p53 have been proposed over the last decade [34]. As peptide vaccine therapy, the wild-type p53 peptide is well preserved in mutant p53 because most mutations in the p53 gene are missense mutations, and are considered to be one of the attractive targets as a cancer antigen. The frequencies of the CTL response against HLA-A24-restricted $p53_{161}$ investigated by the ELISPOT assay in head and neck carcinoma and hepatocellular carcinoma were shown to be 35 and 10 %, respectively [35, 36]. Although the frequency of 7 % in our study is lower, given the difference according to the primary tumor site or balance between sensitivity and specificity, induced CTLs showed cytotoxic activity against pancreatic adenocarcinoma cell lines, which suggested that p53 may be an attractive target in patients with pancreatic cancer.

hTERT is widely overexpressed in various cancer cells including pancreatic cancer [37], which is consistent with our results. A clinical trial demonstrated that GV1001, a HLA class II epitope corresponding to the hTERT (611–626) fragment, was immunogenic in pancreatic cancer patients [38]. Another previous study evaluating T cell responses to several hTERT epitopes in patients with hepatocellular carcinoma [39] demonstrated that $hTERT_{461}$- and $hTERT_{324}$-specific CTLs were induced in 5 (6.9 %) and 9 (12.5 %) of 72 patients, respectively. In the current study, these frequencies were equivalent and the killing of pancreatic cancer cell lines was demonstrated, which suggested that these epitopes also had immunogenicity in pancreatic cancer patients.

Peptide vaccine therapies using $WT\text{-}1_{235}$ and $VEGFR2_{169}$ combined with gemcitabine have already been conducted in pancreatic adenocarcinoma patients [23, 24]. We clarified that $WT\text{-}1_{235}$- and $VEGFR2_{169}$-specific CTLs induced from PBMCs showed cytotoxicity for human pancreatic cancer cell lines, and the results of further investigations are anticipated.

We performed phenotypic analysis of TAA-derived epitope-specific T cells to determine the most appropriate epitope for immunotherapy in patients with pancreatic adenocarcinoma. Epitope-specific $tetramer^+$ cells in PBMCs were also found in patients without IFN-γ ELISPOT responses, which was consistent with the findings of previous studies [39, 40] and suggested the existence of dysfunctional epitope-specific T cells. Epitope-specific $tetramer^+$ cells were also identified at a very low frequency in PBMCs from healthy volunteers and increased in CTLs induced with TAA-derived peptides, which was also consistent with previous studies in which TAA-specific $tetramer^+$ T cells were detectable in samples from healthy donors [41] or the in vitro stimulation of PBMCs with the epitopes derived from TAA could induce TAA-specific CTLs in healthy volunteers [42], even though the precise mechanism has not yet been clarified. Phenotypic analysis showed that the frequency of T cells with each memory and effector phenotype depended on the patient and also that peptide-specific memory T cells existed in PBMCs of patients with pancreatic adenocarcinoma. Because T cells with the memory phenotype exert stronger antitumor effects by secondary stimulation with the antigen, our results suggest that an additional immunological approach such as that consisting of a TAA-derived protein or peptide, recombinant virus, and engineered tumor cells to boost T cell function may be useful to enhance host antitumor immune responses.

Another purpose of this study was to identify the factors influencing immune responses. Our results suggested that the frequencies of the lymphocyte subsets in peripheral leukocytes were very important in the induction of TAA-specific CTLs. Although the relationship between cancer, inflammation, and immunity has already been documented [43], the precise mechanism has yet to be fully understood. One of the speculated reasons why PBMC from patients with lymphocytopenia could not induce a good immune response in our study is that the release of inhibitory immunological cytokines such as transforming growth factor β or IL-10 from pancreatic adenocarcinoma tissue decreases lymphocyte counts and impairs the function of lymphocytes both systemically and in the microenvironment [44]. It was also reported that lymphocyte counts and CTL responses were prognostic markers in advanced cancer cases receiving peptide vaccine therapy [45, 46]. Our results showing a correlation between the T cell response and outcomes in pancreatic adenocarcinoma patients corresponded to these previous findings, which indicate that restricting the objective to those with an adequate lymphocyte subset could lead to a clinical trial with favorable outcomes.

A limitation of this study was the lack of data for the clinical response. Tumor shrinkage or survival benefits are not always observed in all patients who exhibit immune responses. Further, clinical studies using peptides that could induce TAA-specific CTLs are needed to confirm our findings.

In conclusion, we simultaneously compared T cell responses to various TAA-derived epitopes in patients with pancreatic adenocarcinomas; our results suggested that $p53_{161}$, $hTERT_{461}$, $WT\text{-}1_{235}$, and $VEGFR2_{169}$ were the most suitable epitopes for cancer vaccine therapy.

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Conflict of interest The authors do not have any conflict of interest.

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New Susceptibility and Resistance HLA-DP Alleles to HBV-Related Diseases Identified by a Trans-Ethnic Association Study in Asia

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Abstract

Previous studies have revealed the association between SNPs located on human leukocyte antigen (*HLA*) class II genes, including *HLA-DP* and *HLA-DQ*, and chronic hepatitis B virus (HBV) infection, mainly in Asian populations. *HLA-DP* alleles or haplotypes associated with chronic HBV infection or disease progression have not been fully identified in Asian populations. We performed trans-ethnic association analyses of *HLA-DPA1*, *HLA-DPB1* alleles and haplotypes with hepatitis B virus infection and disease progression among Asian populations comprising Japanese, Korean, Hong Kong, and Thai subjects. To assess the association between *HLA-DP* and chronic HBV infection and disease progression, we conducted high-resolution (4-digit) *HLA-DPA1* and *HLA-DPB1* genotyping in a total of 3,167 samples, including HBV patients, HBV-resolved individuals and healthy controls. Trans-ethnic association analyses among Asian populations identified a new risk allele *HLA-DPB1*09:01* ($P = 1.36 \times 10^{-6}$; OR = 1.97; 95% CI, 1.50–2.59) and a new protective allele *DPB1*02:01* ($P = 5.22 \times 10^{-6}$; OR = 0.68; 95% CI, 0.58–0.81) to chronic HBV infection, in addition to the previously reported alleles. Moreover, *DPB1*02:01* was also associated with a decreased risk of disease progression in chronic HBV patients among Asian populations ($P = 1.55 \times 10^{-7}$; OR = 0.50; 95% CI, 0.39–0.65). Trans-ethnic association analyses identified Asian-specific associations of *HLA-DP* alleles and haplotypes with HBV infection or disease progression. The present findings will serve as a base for future functional studies of HLA-DP molecules in order to understand the pathogenesis of HBV infection and the development of hepatocellular carcinoma.

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Introduction

Hepatitis B virus (HBV) infection is a major global health problem, resulting in 0.5–1.0 million deaths per year [1]. The prevalence of chronic HBV infection varies. About 75% of the chronic carriers in the world live in Southeast Asia and East Pacific [2]. Due to the introduction of vaccination programs, the prevalence of HBV infection in many countries has gradually been decreasing with consequent decreases in HBV-related hepatocellular carcinoma (HCC) [3]. Although some HBV carriers spontaneously eliminate the virus, about 10–15% of carriers develop liver cirrhosis (LC), liver failure and HCC [4]. Moreover, the progression of liver disease was revealed to be associated with the presence of several distinct mutations in HBV infections [5]. Genetic variations in *STAT4* and *HLA-DQ* genes were recently identified as host genetic factors in a large-scale genome-wide association study (GWAS) for HBV-related HCC in China [6].

With regard to the genes associated with susceptibility to chronic HBV infection, *HLA-DP* and *HLA-DQ* genes were identified by GWAS in Japanese and Thai populations in 2009 [7] and 2011 [8], respectively. In addition, our previous GWAS confirmed and identified the association of SNP markers located on *HLA-DPA1* (rs3077) and *HLA-DPB1* (rs9277535) genes with susceptibility to chronic hepatitis B (CHB) and HBV clearance in Japanese and Korean subjects[9]. The significant associations of *HLA-DP* with CHB and HBV clearance have mainly been detected in Asian populations, such as Japanese [8,9], Thai [7], Chinese [10–12], and Korean [9]. In 2012, the association between *HLA-DPA1* gene SNPs and persistent HBV infection was replicated in a Germany non-Asian population for the first time; however, this showed no association with HBV infection [13]. These results seem to be explained by the fact that allele frequencies of both rs3077 (0.155, 0.587 and 0.743 for C allele, on HapMap CEU, JPT, and YRI) and rs9277535 (0.261, 0.558 and 0.103 for G allele, on HapMap CEU, JPT, and YRI) are markedly different between populations. Moreover, the previous study showed that HBsAg seropositivity rates were higher in Thailand and China (5–12%) than in North America and Europe (0.2–0.5%) [2]. These results suggest that comparative analyses of *HLA-DP* alleles and haplotypes in Asian populations would clarify key host factors of the susceptible and protective *HLA-DP* alleles and haplotypes for CHB and HBV clearance. Here, we performed trans-ethnic analyses of *HLA-DP* alleles and haplotypes in Asian populations comprising Japanese, Korean, Hong Kong and Thai individuals. The findings from this study will serve as a base for future functional studies of HLA-DP molecules.

Results

Characteristics of studied subjects

The characteristics of a total of 3,167 samples, including Japanese, Korean, Hong Kong and Thai subjects, are shown in Table 1. Each population included three groups of HBV patients, resolved individuals and healthy controls. The clinical definitions of HBV patients and resolved individuals are summarized in Materials and Methods. Some of the Japanese and all of the Korean samples overlapped with the subjects in our previous study [9,14].

We performed genotyping for *HLA-DPA1* and *HLA-DPB1* in all 3,167 samples, and a total of 2,895 samples were successfully genotyped. The characteristics of successfully genotyped samples are shown in Table S1.

Association of *HLA-DPA1* and *HLA-DPB1* alleles in Asian populations

As for a general Asian population, including 464 Japanese, 140 Korean, 156 Hong Kong, and 122 Thai subjects, five *HLA-DPA1* alleles and twenty-four *HLA-DPB1* alleles were observed (Table S2). The frequencies of *HLA-DPA1* and *HLA-DPB1* alleles were similar between Japanese and Korean subjects. On the other hand, the number of alleles with frequencies of 1–2% was larger in Hong Kong and Thai populations, despite the small sample size. Although the frequencies of *HLA-DP* alleles varied in Asian populations, *HLA-DPB1*05:01* was the most prevalent with over 30% in all populations.

The associations of *HLA-DPA1* and *HLA-DPB1* alleles with chronic HBV infection (i.e., comparison between HBV patients and healthy controls) are shown in Table S2. To avoid false positives caused by multiple testing, the significance levels were corrected based on the numbers of *HLA-DPA1* and *HLA-DPB1*

Table 1. Number of individuals in this study.

Population	Japanese	Korean	Hong Kong	Thai
Total number of samples	1,291	586	661	629
HBV patients	489	340	281	390
IC	114	-	-	-
CH	147	175	187	198
AE	21	-	-	-
LC	38	-	-	-
HCC	169	165	94	192
Mean age (y)	57.1	44.7	57.9	52.0
(min-max)	(20–84)	(18–74)	(32–86)	(21–84)
Gender (M/F)	338/151	265/75	239/42	289/101
Resolved individuals*	335	106	190	113
HCV (−)	249	106	190	113
HCV (+)	86	-	-	-
Mean age (y)	59.7	43.1	40.0	48.2
(min-max)	(18–87)	(12–66)	(18–60)	(39–66)
Gender (M/F)	173/162	61/45	113/77	83/30
Healthy controls	467	140	190	126
Mean age (y)	39.0**	33.7	26.2	46.6
(min-max)	(23–64)	(1–59)	(16–60)	(38–79)
Gender (M/F)	370/97	67/73	87/103	73/53

Abbreviation: IC, Inactive Carrier; CH, Chronic Hepatitis; AE, Acute Exacerbation; LC, Liver Cirrhosis; HCC, Hepatocellular Carcinoma.
* Resolved individuals were HBsAg negative and HBcAb positive.
** 419 of 467 healthy controls were de-identified, without information on age.
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alleles in the focal population. Briefly, the significance level was set at 0.05/(# of observed alleles at each locus) in each population (see Materials and Methods). With regard to high-risk alleles of *HLA-DPA1*, the most prevalent allele *HLA-DPA1*02:02* was significantly associated with susceptibility to HBV infection in Japanese ($P = 3.45 \times 10^{-4}$; OR = 1.39; 95% CI, 1.16–1.68) and Korean subjects ($P = 2.66 \times 10^{-5}$; OR = 1.89; 95% CI, 1.39–2.58), whereas this association was not observed in Hong Kong or Thai subjects. The association of *HLA-DPA1*02:01* with susceptibility to HBV infection was significant only in Japanese ($P = 2.61 \times 10^{-7}$; OR = 1.88; 95% CI, 1.46–2.41). The significant association of *HLA-DPA1*01:03* with protection against HBV infection was commonly observed among four Asian populations (Table S2). The pooled OR and 95% CI were 0.51 and 0.41–0.63, respectively in a meta-analysis ($P = 3.15 \times 10^{-10}$) (Fig. S1A).

As shown in Table S2, *HLA-DPB1* shows higher degree of polymorphism than *HLA-DPA1*. The most common allele in Asian populations, *HLA-DPB1*05:01*, was significantly associated with HBV susceptibility in both Japanese and Korean subjects. Although *HLA-DPB1*05:01* showed no significant association in the Hong Kong and Thai populations, the same direction of association (i.e., HBV susceptibility) was observed. Meta-analysis of the four populations revealed a significant association between *HLA-DPB1*05:01* and susceptibility to HBV infection ($P = 1.51 \times 10^{-4}$; OR = 1.45; 95% CI, 1.19–1.75) (Fig. S1B). The frequency of *HLA-DPB1*09:01* was significantly elevated in Japanese HBV patients (15.7%) as compared with healthy controls (8.7%) ($P = 3.70 \times 10^{-6}$; OR = 1.94; 95% CI, 1.45–2.62), and this association was most significant (i.e., the smallest P value) in the Japanese population. Because of lower allele frequencies of *HLA-DPB1*09:01* or lack of statistical power in the other populations, no significant associations were observed. A common allele in Thai subjects, *HLA-DPB1*13:01*, was significantly associated with susceptibility to HBV infection ($P = 2.49 \times 10^{-4}$; OR = 2.17; 95% CI, 1.40–3.47) with the same direction of associations in Japanese and Hong Kong (OR = 1.52 and 1.40, respectively).

*HLA-DPB1*04:02* was identified as the most protective allele for HBV infection in Japanese ($P = 1.59 \times 10^{-7}$; OR = 0.37; 95% CI, 0.24–0.55) and Korean subjects ($P = 1.27 \times 10^{-7}$; OR = 0.19; 95% CI, 0.10–0.38). Both *HLA-DPB1*02:01* and *HLA-DPB1*04:01* were also significantly associated with protection in the Japanese population, and the former was significantly associated with protection in Hong Kong subjects ($P = 9.17 \times 10^{-4}$; OR = 0.49; 95% CI, 0.32–0.76). This common allele among four Asian populations, *HLA-DPB1*02:01*, showed a significant association with protection against HBV infection ($P = 5.22 \times 10^{-6}$; OR = 0.68; 95% CI, 0.58–0.81) in a meta-analysis (Fig. S1B).

The frequencies of associated *HLA-DP* alleles in a comparison of HBV patients with healthy controls (Table S2) or with HBV-resolved individuals (Table S3) were similar in all four Asian populations. In the Japanese population, the associations of susceptible and protective *HLA-DPB1* alleles to chronic HBV infection seem weaker in the comparison of HBV patients with HBV-resolved individuals than in the comparison of HBV patients with healthy controls. Moreover, the results of association analyses showed no difference in the comparison of HBV patients with HBV-resolved individuals, including or excluding HCV positive individuals (Table S3). In contrast, the association became stronger in the comparison of HBV patients with HBV-resolved individuals among the Korean subjects. The protective allele *HLA-DPB1*04:01* was also identified to have a strong association with HBV clearance in Hong Kong subjects (Table S3). Moreover, in Hong Kong subjects, the *HLA-DPB1*05:01* associated with the risk for HBV infection showed lower frequency in HBV-resolved individuals (42.9%) than in the healthy controls (48.1%), which accounts for a strong association in the comparison of HBV patients with HBV-resolved individuals ($P = 6.24 \times 10^{-3}$; OR = 1.64; 95% CI, 1.14–2.36). Although the number of samples was insufficient, *HLA-DP*100:01* showed a significant association with protection against HBV infection in the Hong Kong population ($P = 3.05 \times 10^{-6}$; OR = 0.03; 95% CI, 0.0007–0.20).

Table 2. Association of number of *DPB1*02:01* alleles (i.e., 0, 1 or 2) with disease progression in CHB patients assessed by multivariate logistic regression analysis adjusted for age and sex.

Population	P value	OR (95% CI)
Japanese	0.000177	0.47 (0.32–0.70)
Korean	0.025358	0.55 (0.33–0.93)
Hong Kong	0.040842	0.46 (0.22–0.97)
Thai	0.087782	0.58 (0.31–1.08)
All*	1.55×10^{-7}	0.50 (0.39–0.65)

*Population was adjusted using dummy variables.

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As for disease progression in CHB patients among Asian populations, a protective effect of *HLA-DPB1*02:01* on disease progression was observed in the Japanese ($P = 4.26 \times 10^{-5}$; OR = 0.45; 95% CI, 0.30–0.67) and Korean populations ($P = 8.74 \times 10^{-4}$; OR = 0.47; 95% CI, 0.29–0.75) (Table S4). Multivariate logistic regression analysis adjusted for age and sex revealed that the number of *DPB1*02:01* alleles (i.e., 0, 1, or 2) was significantly associated with disease progression in CHB patients in Japanese ($P = 1.77 \times 10^{-4}$; OR = 0.47; 95% CI, 0.32–0.70) (Table 2). Moreover, protective effects of *DPB1*02:01* on disease progression in Asian populations ($P = 1.55 \times 10^{-7}$; OR = 0.50; 95% CI, 0.39–0.65) were detected in a multivariate logistic regression analysis adjusted for age, gender, and population (Table 2).

Associations of *DPA1-DPB1* haplotypes in Asian populations

The estimated frequencies of *HLA DPA1-DPB1* haplotypes are shown in Table S5. The most frequent haplotype among the four Asian populations was *DPA1*02:02-DPB1*05:01*. The number of haplotypes with low frequencies of 1–2% was 10 in both Japanese and Korean subjects, whereas more haplotypes appeared with frequencies of 1–2% in Hong Kong and Thai subjects. The associations of *DPA1-DPB1* haplotypes with HBV infection are shown in Table S5. In the Japanese population, *DPA1*02:01-DPB1*09:01* showed the most significant association with susceptibility to HBV infection ($P = 3.38 \times 10^{-6}$; OR = 1.95; 95% CI, 1.46–2.64). The most common haplotype in the four Asian populations, *DPA1*02:02-DPB1*05:01*, was found to be significantly associated with susceptibility to HBV infection in the Japanese and Korean subjects ($P = 7.40 \times 10^{-4}$; OR = 1.37; 95% CI, 1.14–1.66 for Japanese, and $P = 4.50 \times 10^{-6}$; OR = 2.02; 95% CI, 1.48–2.78 for Korean). In the Thai subjects, *HLA-DPB1*13:01* was the most significant risk allele for HBV infection (Table S2); however, no significant associations were found for the three different haplotypes bearing *HLA-DPB1*13:01*: *DPA1*02:01-DPB1*13:01*, *DPA1*02:02-DPB1*13:01*, and *DPA1*04:01-DPB1*13:01*,indicating that the association of *HLA-DPB1*13:01* with susceptibility to HBV infection did not result from a specific *DPA1-DPB1* haplotype or combination with a specific *DPA1* allele.