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# Clinical Trial of a Cancer Vaccine Targeting VEGF and KIF20A in Advanced Biliary Tract Cancer

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**Abstract.** *Background: As the prognosis of biliary tract cancer (BTC) is extremely poor and treatment options are limited, new treatment modalities are urgently needed. We designed a phase II clinical trial to investigate the immune responses and clinical benefits of OCV-C01, an HLA-A*24:02–restricted three-peptide cancer vaccine targeting VEGFR1, VEGFR2, and KIF20A. Patients and Methods: Participants were patients with advanced BTC who had unresectable tumours and were refractory to standard chemotherapy. OCV-C01 was injected weekly until the discontinuance criteria were met. Results: Six participants, including four patients positive for HLA-A*24:02, were enrolled in this study for assessment of efficacy. Four out of six patients exhibited vaccine-specific T-cell responses to one or more of three antigens. Log–rank tests revealed that vaccine-specific T cell responses contributed significantly to overall survival. Conclusion: The cancer vaccine had positive effects on survival, indicating that this approach warrants further clinical studies.*

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Biliary tract cancer (BTC) is often advanced and unresectable at the time of diagnosis, and refractory cancers recur in 50-80% even after curative resection (1). Hence, post-recurrence treatment plays a large role in patient care. The first-line standard therapy for unresectable BTC is gemcitabine (GEM) + cisplatin, with a median survival time (MST) of 11.7 months (2). Standard second-line therapy has not yet been established. Therefore, new treatment modalities are urgently needed.

Several reports have described clinical studies of immunotherapies for advanced BTC. A clinical trial of the combination of GEM or GC with the WT1 peptide vaccine has been conducted, but no clear clinical benefit was observed (3, 4). On the other hand, a phase I trial using a multi-peptide vaccine containing KIF20A, which is also used in the trial described here, achieved a median survival of 9.7 months (5). This vaccine induced KIF20A-specific immune responses in eight of nine patients with BTC. To extend the findings of the previous clinical trial, we conducted a phase II clinical trial of the HLA-A*24:02–restricted cocktail peptide cancer vaccine OCV-C01 (VEGFR1, VEGFR2, and KIF20A) in patients with advanced recurrent BTC who were refractory to standard therapy.

VEGF-A binds to two receptor tyrosine kinases, namely VEGFR1 and VEGFR2. It plays a major role in tumour vascularisation by promoting proliferation of vascular endothelial cells and increasing vascular permeability, while its expression is elevated in most tumours (6). Expression of VEGF-A is induced by hypoxia, hypoglycaemia, and various growth factors and oncogenes (6). Therefore, induction of cytotoxic T lymphocytes (CTLs) targeting the vascular endothelial cells that express VEGFR-1 and VEGFR-2 is

expected to inhibit angiogenesis, cancer cell proliferation, and metastasis. Furthermore, KIF20A is a tumour-specific antigen (oncoantigen) that is expressed at high levels in pancreatic cancer and cholangiocarcinoma tissues but at lower levels in normal tissues (7). The HLA-A*24:02–restricted epitope peptide (OCV-105) derived from KIF20A can induce a CTL that specifically damages KIF20A-expressing cells (8).

When tumour-specific antigens and tumour neovascular endothelial cells are adopted as targets for cancer peptide vaccine, does it lead to a combined effect? To investigate this question, an adoptive T-cell transfer experiment was performed. In a tumour-bearing model where mice were intradermally transplanted with B16 melanoma, the authors investigated the *in vivo* antitumour effect of simultaneous transfer of genetically engineered syngeneic T cells expressing a chimeric antigen receptor targeting VEGFR2 and T cells specific for tumour antigens. A synergistic inhibition of tumour growth was observed when T cells targeting VEGFR2 and tumour-specific antigens were administered in combination (9). Theoretically, by increasing the functionality of tumour vessels and fostering an immunosupportive tumour microenvironment, vascular normalisation approaches might not only improve the development of anticancer immunity, but also decrease the required doses of cytotoxic therapies and thereby decrease the adverse effects of combinations incorporating immunotherapies (10-12). Therefore, to develop a reasonable cancer vaccine strategy we have focused on OCV-C01, which targets both tumour angiogenesis and an oncoantigen. Although this was a single-group study, it was designed to compare the clinical effects of HLA-matched and non-matched groups to obtain the proof of concept for this vaccine by opening HLA after the trial. The immune responses to the vaccine, and the clinical result in both the HLA-matched and HLA-unmatched groups, suggested the possibility of efficacy with alleles other than the target allele, HLA-A*24:02. We also consider the possibility of future development of this vaccine to target alleles other than HLA-A*24:02.

## Patients and Methods

*Study design*. This trial was a phase II, single-group, open-label, single-centre study. We investigated the efficacy and safety of the cocktail peptide cancer vaccine OCV-C01 in patients with previously treated refractory advanced BTC. The study was approved by the Institutional Ethical Review Board of Kyushu University (#CTR044-001) and registered with University hospital Medical Information Network-Clinical Trial Registry (UMIN-CTR) (UMIN000012778). Prior to participating in this clinical trial, written informed consent was obtained from all patients, and screening tests were performed. OCV-C01 was administered once a week at 1.0 ml, until the criteria for discontinuation of the study drug were met; the start date of study drug administration was defined as Day 1. The discontinuation criteria of the study drug were as follows: when administration of the investigational drug cannot be started by the 15$^{th}$ day with the registration date as the 1$^{st}$ day; when a clear exacerbation of the underlying disease is observed; when a diffuse alveolar damage pattern is confirmed from chest imaging findings; when a patient with a lung lesion has pleural effusion that is difficult to be controlled by the investigator; when an increase in pleural effusion is observed in a patient with carcinomatous pleurisy; when an adverse event occurs that makes it difficult to continue administration of the investigational drug; when the investigational drug administration is skipped three times continuously; when pregnancy is found; when any matter that violates the following administration start criteria occurs. The following period has to be passed from the end date of pretreatment to the start date of administration of this investigational drug. Namely, 1) 14 days for chemotherapeutic drugs; 2) 28 days for domestic unapproved drug besides 56 days for antibody formulation; 3) 28 days for radiation therapy; 4) 14 days for laparotomy; 5) 14 days for systemic corticosteroids; when the subject wishes to discontinue administration of the investigational drug; when a patient withdraws the consent; when continuous medical care becomes difficult due to patient's relocation, hospital transfer, or very busy schedule, *etc*; when it turns out the patient was not eligible even after enrollment; when the investigator determines that it is necessary to discontinue administration of the investigational drug. The period of study drug administration ran from Day 1 to Day 365. Because at least 13 subjects with HLA-A*24: 02 were expected, 40 patients with unknown HLA (as OCV-C01 administration cases) were set as the target number of cases. The duration of the study was as follows: October 7, 2014 (date of first participant consent); December 8, 2015 (date of the decision by the efficacy and safety committee to discontinue the study); July 26, 2016 (last observation day of the last participant). The primary efficacy endpoint was overall survival (OS). The secondary evaluation items were as follows: efficacy endpoints, progression-free survival (PFS) and tumour regression effect; safety endpoints, rate of adverse events and rate of serious adverse events; exploratory endpoints, OCV-C01–specific CTLs.

*Patient eligibility*. Disease inclusion criteria were as follows: diagnosis of BTC (intrahepatic bile duct cancer, extrahepatic bile duct cancer, gallbladder cancer, papillary cancer); histological diagnosis of "adenocarcinoma (papillary adenocarcinoma, tubular adenocarcinoma), adenocarcinoma in the case of extrahepatic cholangiocarcinoma, gallbladder cancer, papillary cancer", or "adenocarcinoma in the case of intrahepatic cholangiocarcinoma"; presence or absence of measurable lesions did not matter; Stage II-IV patients with unresectable BTC (in case of recurrence, the stage at the time of initial onset does not matter); refractory to the gemcitabine-containing regimen (including recurrence) or intolerant to the gemcitabine-containing regimen (could not be administered for medical reasons); radiation therapy not expected to prolong survival; PS (ECOG) of either 0 or 1; age at time of obtaining consent >20 years; major organ functions are maintained and meet all of the following criteria: 1) white blood cell count ≥2,000/mm$^3$, 2) neutrophil count ≥1,000/mm$^3$, 3) haemoglobin ≥8.0 g/dL, 4) platelet count ≥5.0×10$^4$/mm$^3$, 5) AST≤200 IU/l, 6) ALT≤200 IU/l, 7) serum creatinine ≤1.5 mg/dl. Exclusion criteria were as follows: history of cancer immunotherapy (activated lymphocyte therapy,

dendritic cell therapy, cancer vaccine therapy, *etc*.); double cancer in less than a year (carcinoma *in situ* and intramucosal cancer lesions could still be registered); strong concern about bleeding due to invasion of the underlying disease into other organs; history or complication of interstitial pneumonia or pulmonary fibrosis; moderate or higher ascites and pleural effusion that are difficult to control; pericardial fluid requiring fine needle drainage; severe infection (including suspicion); brain metastasis (including suspicion); severe mental illness or severe neuropathy; uncontrolled heart, lung, kidney, or liver disease; grade 4 events (including abnormal laboratory test values); history of myocardial infarction, severe unstable angina, coronary/peripheral artery bypass surgery, congestive heart failure, cerebrovascular accident, pulmonary embolism, deep vein thrombosis, and other serious thromboembolism within 12 months before the start of study drug administration; traumatic lesions that have not healed (including fractures); bleeding diathesis (including aneurysms that could be fatal due to rupture) or excessive coagulopathy; requirement for continued administration of antithrombotic drugs other than aspirin; uncontrolled hypertension; heart failure in need of treatment; requirement for systemic administration of the following drugs during the study drug administration period: 1) corticosteroids (continuous administration at least once every two weeks), 2) immunosuppressants, immunostimulants, 3) G-CSF/M-CSF, 4) erythropoietin; participating in other clinical trials or clinical studies (excluding trials without intervention); HLA known in advance; pregnant or breastfeeding that cannot be stopped from the start date of study drug administration to 120 days after the final administration of the study drug; patient or partner has no intention of contraception; and patients who were judged to be ineligible by the investigator.

*Peptides and vaccination*. OCV-C01 is a ready-to-use injection consisting of the following two types of vials: Vial 1: Lyophilised formulation containing elpamotide, OCV-101 and OCV-105 as active ingredients; Vial 2: Incomplete Freund's adjuvant (Montanide ISA 51 VG). The origins and sequences of the three peptides, all of which bind the HLA-A24 molecule, are as follows: elpamotide, VEGFR2, RFVPDGNRI; OCV-101, VEGFR1, SYGVLLWEI; and OCV-105, KIF20A, KVYLRVRPLL. At the time of use, the lyophilised preparation was dissolved in 1 mL of water for injection, and then mixed with incomplete Freund's adjuvant at a volume of 1:1 to give an emulsion. One ml of emulsion contained 2 mg of elpamotide, 2 mg of OCV-101, and 3 mg of OCV-105. The investigational drug was administered at a dose of 1.0 ml once a week for 4 weeks by subcutaneous injection in the axilla or groin.

*CTL assay (enzyme-linked ImmunoSpot)*. A CTL assay was performed using PBMCs collected on Days 1, 29, 57, and Day 180. Vaccine-specific T-cell response was estimated by enzyme-linked ImmunoSpot (ELISPOT) following *in vitro* sensitisation (IVS), as described previously (13, 14) with some modifications. Frozen PBMCs from patients were thawed together, and viability was confirmed at >90%. PBMCs ($0.5\times10^6$/ml) were cultured for 2 weeks at 37˚C with 10 μg/ml of each peptide and 60 IU/ml of IL-2 (Novartis, Emeryville, CA, USA). The peptides were added to the culture at days 0 and 7. After $CD4^+$ cells were depleted using a Dynabeads CD4-positive isolation kit (Invitrogen, Carlsbad, CA, USA), an IFN-γ ELISPOT assay was performed using the Human MabTech PLUS kit (Nacka Strand, Sweden). Briefly, human TISI cells ($2\times10^5$ cells/ml) from the B-lymphoblastoid cell line, which is positive for HLA-A*2402 (IHWG Cell and Gene Bank, Seattle, WA, USA), were incubated overnight with 20 μg/ml of each peptide, and then any residual peptides were washed out to prepare peptide-pulsed TISI cells (Stimulator cells). Prepared CD4- cells (Responder cells) were cultured in 96-well plates (Millipore, Bedford, MA, USA) at 37˚C overnight with peptide-pulsed TISI cells ($2\times10^4$ cells/well) at R/S (Responder/Stimulator) ratios of 1.0, 0.5, 0.25, and 0.125. Non-peptide–pulsed TISI cells were used as negative stimulator controls. For the HLA-A*24:02–unmatched-group, ELISPOT assay was performed without Stimulator cells (Dump assay). PBMCs after IVS culture and depletion of $CD4^+$ T cells were cultured with or without 2.5 mg/ml of peptide at 37˚C under 5% $CO_2$ overnight. To confirm the productivity of IFN-γ responder cells, as a positive control, cells were stimulated with 25 ng/mL of PMA (phorbol 12-myristate 13-acetate, Sigma-Aldrich, St Louis, MO, USA) and 1 μg/ml of ionomycin (Sigma-Aldrich) overnight, and then subjected to the IFN-γ ELISPOT assay ($2.5\times10^3$ cells/well) in the absence of stimulator cells. All ELISPOT assays were performed in triplicate wells. The plates were analysed on an ImmunoSpot S5 Versa automated ELISPOT reader using the ImmunoSpot Professional Software Version 5.0 (Cellular Technology Ltd, Shaker Heights, OH, USA). Measurements were performed in triplicate in the peptide pulse group and negative control group for each time point and the median values were then calculated. In addition, measured values (spots) with an R/S ratio of 1.0 or 0.5 were used to calculate the representative values of each group. Other than that, the value was set as not available (N.A.). Specific spot (hereinafter, SS) was calculated for each time point of each case as follows: SS=Representative value of peptide pulse group-Representative value of negative control group. When SS had a negative value it was read as 0. Using the SS calculated by the above formula, % specific spot (hereinafter, %SS) was calculated at each time point of each case as follows: %SS=(SS/Representative value of peptide pulse group) ×100. The value was defined as positive only if both of the following criteria were met at each time point other than Day T1, and otherwise was negative. Criterion 1: % SS at each time point is larger than the pre-administration value (Day T1). Criterion 2: The representative value of the peptide pulse group is larger than the range of the representative value of the negative control (with standard deviation added).

*TCR sequencing*. Total RNAs were extracted from PBMCs after $CD4^+$ T-cell depletion following IVS culture using the RNeasy mini kit (QIAGEN, Hilden, Germany). cDNAs with common 5'-RACE adapters were synthesised using SMARTScribe Reverse Transcriptase (Clontech, Mountain View, CA, USA). cDNA libraries of *TRAV* and *TRBV* were subjected to sequencing on the Illumina MiSeq platform using 600 cycles (MiSeq Reagent Kit V3; Illumina, San Diego, CA, USA). To identify V-(D)-J segments in individual *TRAV* and *TRBV* sequencing reads, each of the sequence reads were mapped to the reference sequences in IMGT/GENE-DB (15). A conserved cysteine encoded in the 3'portion of the V segment and a conserved phenylalanine in the 5'portion of the J segment was used to identify the amino acid sequences of CDR3 in *TRAV* and *TRBV*.

*TCR repertoire analysis*. To visualise TCR sequencing data, we generated pie charts using Microsoft Excel. Pie charts show the frequency of TCR clonotypes in PBMCs after $CD4^+$ T-cell depletion following IVS culture. The top 10 high-frequency TCR clonotypes

Table I. *Individual patient characteristics*.

| Patient | Gender | Age | PS | Diseases | | Prior therapy | | Post therapy |
|---|---|---|---|---|---|---|---|---|
| | | | | Primary | Metastases | Surgery | Chemotherapy | |
| OCV-001 | M | 71 | 1 | EBD | Liver, Lung, Skin, LNs | + | GEM, CDDP, S-1 | – |
| OCV-003 | F | 55 | 0 | IBD | Liver, LNs | – | GEM, CDDP | S-1 |
| OCV-004 | F | 51 | 0 | EBD | Pancreas, Lung | + | GEM, CDDP, S-1 | FOLFOX |
| OCV-005 | F | 70 | 0 | EBD | Liver | – | GEM, CDDP, S-1 | – |
| OCV-006 | F | 54 | 1 | IBD | Liver | – | GEM, CDDP, S-1 | – |
| OCV-007 | F | 68 | 1 | IBD | Liver | + | GEM, CDDP | GEM, S-1 |
| OCV-008 | F | 85 | 1 | IBD | Liver | – | GEM | Nivolumab, ALI |

PS, Performance status; IBD, intrahepatic bile duct cancer; EBD, extrahepatic bile duct cancer cancer; LN, lymph nodes. GEM, gemcitabine; CDDP, cisplatin; ALI, activated lymphocyte infusion.

are shown individually. The light grey zone indicates the sum of TCR clonotypes outside the top 10. To estimate TCR repertoire diversity, the inverse Simpson's index was calculated based on the following equation:

$$DS=i=1Kni(ni-1)N(N-1)-1$$

where *K* is the total number of TCR clonotypes, ni is the number of sequence reads for the i-th clonotype, and *N* is the total number of sequence reads in TCR sequencing.

*Immunohistochemical analysis*. The evaluation target site was confirmed using HE-stained tissue slides prepared from the same FFPE block as each evaluation target specimen. In addition, the validity of immunostaining was confirmed for samples prepared from the same FFPE block as the sample to be evaluated using a specimen stained with normal IgG from allogeneic animals (*i.e.*, a negative control) instead of the anti-KIF20A antibody.

*Evaluation target*. Firstly, only clear tumour cells (human BTC) were evaluated by referring to HE-stained specimens, while non-tumour cells (including interstitial cells such as blood vessels) were excluded from the evaluation. Secondly, even if a cell was a tumour cell, if there was a necrotic region or suspected contamination, it was excluded from the evaluation target. Thirdly, if the specimen did not contain clear tumour cells, or if evaluation was not possible due to detachment of the tumour cells, *etc*., it was described as "Not evaluable" (NE). Furthermore, cases where the nucleus was clearly stained were evaluated as specific staining results (specific staining).

*Evaluation criteria*. Specimens with both intensity score and proportion score of 1 or higher were accepted as positive. Concerning the intensity score (I score), the staining intensity was evaluated using a score of 0-3. Score 3 (strongly positive): specific staining was clear even with a 4× objective lens; Score 2 (intermediately positive): specific staining was unclear when observed with a 4× objective lens, but clearly observed with a 10x objective lens; Score 1 (weakly positive): specific staining was unclear with a 10× objective lens, and could only be identified with a 40× objective lens; Score 0 (negative): no specific staining was observed even with a 40× objective lens.

The proportion score (P score) was defined as the proportion of positive cell nuclei in the stained area and was evaluated in the following four stages. All positive cells in the staining area were judged regardless of the I score, and were not limited to tumour cells stained with the same intensity. Score 3 (widely positive): specific staining with an I score ≥1 was observed in 50% or more of the target cells; Score 2 (intermediately positive): specific staining with an I score ≥1 was observed in more than 10% and less than 50% of target cells; Score 1 (weakly positive): specific staining with an I score ≥1 was observed in <10% of the target cells; Score 0 (negative): no specific staining was observed in the target cells (I score=0).

*Statistical methods*

*Analysis population*. The target population for efficacy analysis was the analysis population from all registered cases excluding the following: 1) cases that significantly deviated from the provisions of the study protocol; and 2) cases that dropped out without receiving any treatment with the investigational drug. The target population for safety analysis included all patients who received treatment with the investigational drug even once.

*Analysis of efficacy endpoints*. The overall survival (OS) and progression-free survival (PFS) were analysed. We compared the survival time of the HLA-A*24:02–retaining population who received OCV-C01 therapy to that of a virtual control population who did not receive OCV-C01 therapy. The log–rank test and Harrington–Fleming test were performed by dividing the patients into groups with and without HLA-A*24:02 and those with and without an immune response to OCV-C01. In addition, a survival curve was generated using the Kaplan–Meier method and the median survival time and 95% confidence intervals on both sides were estimated. Tumour regression effect was evaluated based on the Response Evaluation Criteria in Solid Tumor (RECIST) criteria. The evaluation of the best overall response was shown for subjects with measurable lesions during the screening period. Changes in serum tumour marker CA19-9 were also evaluated. The mean value and standard deviation of the amount of change from baseline were calculated for each time point after administration, and a paired t-test was performed. The number of weeks since the start of administration of the investigational drug was plotted on the horizontal axis, and the measured values on the vertical axis.

Table II. *Overall adverse events (AEs).*

| Overall adverse events | No. of patients (%) | | | |
|---|---|---|---|---|
| | Grade 1/2 AEs | Grade 3 AEs | Grade 4 AEs | Grade 5 AEs |
| Vaccine-related AEs | | | | |
| Injection site reaction | 6 (85.7) | 0 | 0 | 0 |
| Headache | 1 (14.3) | 0 | 0 | 0 |
| Discomfort of oral cavity | 1 (14.3) | 0 | 0 | 0 |
| Hot feeling | 1 (14.3) | 0 | 0 | 0 |
| Fever | 1 (14.3) | 0 | 0 | 0 |
| Disease-related AEs | | | | |
| Cytitis | 1 (14.3) | 0 | 0 | 0 |
| Herpes symplex | 1 (14.3) | 0 | 0 | 0 |
| Sepsis | 0 | 0 | 1 (14.3) | 0 |
| Anemia | 1 (14.3) | 0 | 0 | 0 |
| Hypokalemia | 0 | 1 (14.3) | 0 | 0 |
| Hypoalbuminemia | 1 (14.3) | 0 | 0 | 0 |
| Hypophagia | 2 (28.6) | 0 | 0 | 0 |
| Hypercreatininemia | 1 (14.3) | 0 | 0 | 0 |
| Anxiety | 1 (14.3) | 0 | 0 | 0 |
| Insomnia | 1 (14.3) | 0 | 0 | 0 |
| Palpitation | 1 (14.3) | 0 | 0 | 0 |
| Embolism | 1 (14.3) | 0 | 0 | 0 |
| Hypoxia | 0 | 1 (14.3) | 0 | 0 |
| Abdominal distention | 1 (14.3) | 0 | 0 | 0 |
| Abdominal pain | 2 (28.6) | 0 | 0 | 0 |
| Cheilitis | 1 (14.3) | 0 | 0 | 0 |
| Diarrhea | 1 (14.3) | 0 | 0 | 0 |
| Periodonitis | 1 (14.3) | 0 | 0 | 0 |
| Cholangitis | 0 | 1 (14.3) | 0 | 0 |
| Arthralgia | 1 (14.3) | 0 | 0 | 0 |
| Arthritis | 1 (14.3) | 0 | 0 | 0 |
| Dysuria | 1 (14.3) | 0 | 0 | 0 |
| Acute kidney injury | 0 | 0 | 0 | 1 (14.3) |
| Menopausal symptoms | 1 (14.3) | 0 | 0 | 0 |
| Malaise | 1 (14.3) | 0 | 0 | 0 |
| Peripheral edema | 1 (14.3) | 0 | 0 | 0 |
| Weight loss | 2 (28.6) | 0 | 0 | 0 |

Furthermore, vaccine-specific CTLs were evaluated. A list of comprehensive judgments and a summary table were created for each case and each peptide. In addition, the positive rate was calculated for each retention state of HLA-A*24:02 and peptide vaccine. The software used for the statistical analyses in this study was Version 9.4 of SAS for Windows.

## Results

*Profile of patients and toxicities*. The original plan targeted 40 patients with unknown HLA status, including at least 13 HLA-A*24:02–matched cases. However, fewer patients than expected met the eligibility criteria. The efficacy and safety evaluation committee held 20 months after the start of registration decided to discontinue the clinical trial due to insufficient number of registrations. Patient baseline characteristics are shown in Table I. The minimum number of administrations was the second administration of the first course, and the maximum was the first administration of the thirteenth course. The period from the initial administration date to the drug administration discontinuation date was 10-358 days. Of the 7 patients who received the study drug, six were included in the efficacy analysis group, excluding one patient who had a significant deviation from the study protocol (violation of exclusion criteria: history of deep vein thrombosis). Of the population analysed for efficacy, four were positive for HLA-A*24:02 and two were not. Seven patients who received the study drug were included in the safety analysis target population. For adverse events (AEs), a table summarising the number of cases and the rate of cases by grade is shown in Table II. There were 51 AEs in 6 of 7 cases (85.7%): 17 events in three of four cases (75.0%) in the HLA-A*24:02–matched group and 34 events in the

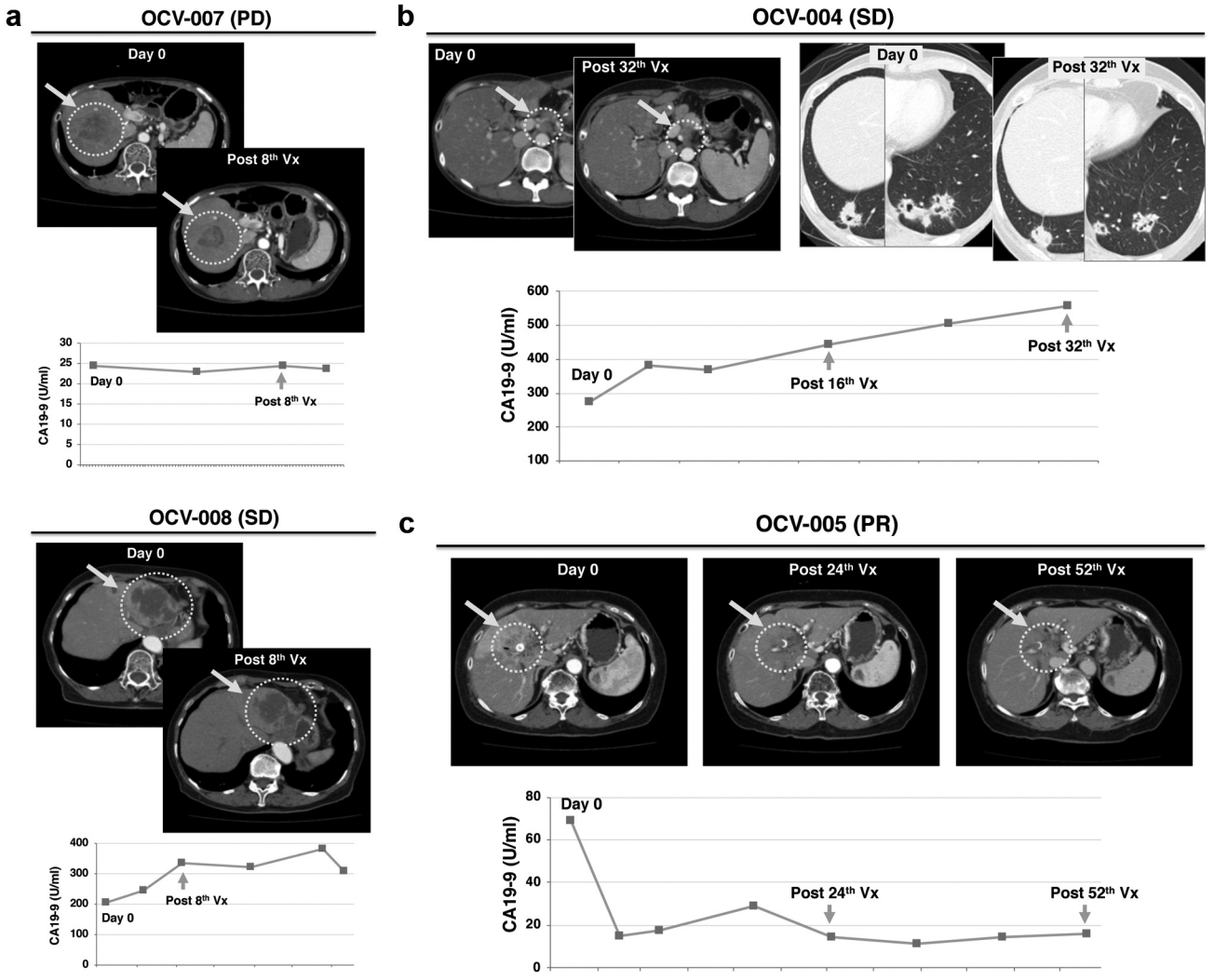


Figure 1. *Clinical responses and tumour markers in patients. Time course of CT imaging of target lesions and CA19-9 as a tumour marker in patients. Arrows and dotted circles show intrahepatic metastases of BTC as target lesions. (a) Cases OCV-007 and -008 from the HLA-A*24:02–matched group; (b) OCV-004 and (c) OCV-005 from the HLA-A*24:02–unmatched group. PD, Progressive disease; SD, stable disease; PR, partial remission; Vx, vaccination.*

three cases (100.0%) of the HLA-A*24:02-unmatched group. AEs that occurred in two or more cases were hyperkalaemia, loss of appetite, abdominal pain, weight loss (2/7 cases), diarrhoea, and fever (3/7 cases). Six of seven patients experienced grade 1 (nine patients) or grade 2 AE at the vaccination site. Other vaccine-related AEs were observed in an HLA-matched case (14.3%) as follows: headache, oropharyngeal discomfort, heat, and fever. During the trial, one patient died of acute kidney injury. Other serious AEs were reported in one patient with hypoxia and in one patient with sepsis and cholangitis (four events). The patient Case 5 was vaccinated for about a year, during which time she developed cholangitis. She presented with bloodstream infections on a blood culture test and was diagnosed with sepsis. However, she was cured by antimicrobial treatment. None of them was judged to be related to the investigational drug. The adverse events that led to the skipping of investigational drug administration were as follows: cholangitis (five events), sepsis, and hypoalbuminemia (one event each).

*Clinical responses*. For patients with HLA-A*24:02, we investigated whether overall survival, which was the primary efficacy endpoint, was longer than in the virtual control population who did not receive OCV-C01 therapy. However, we observed no significant difference. In addition, we observed

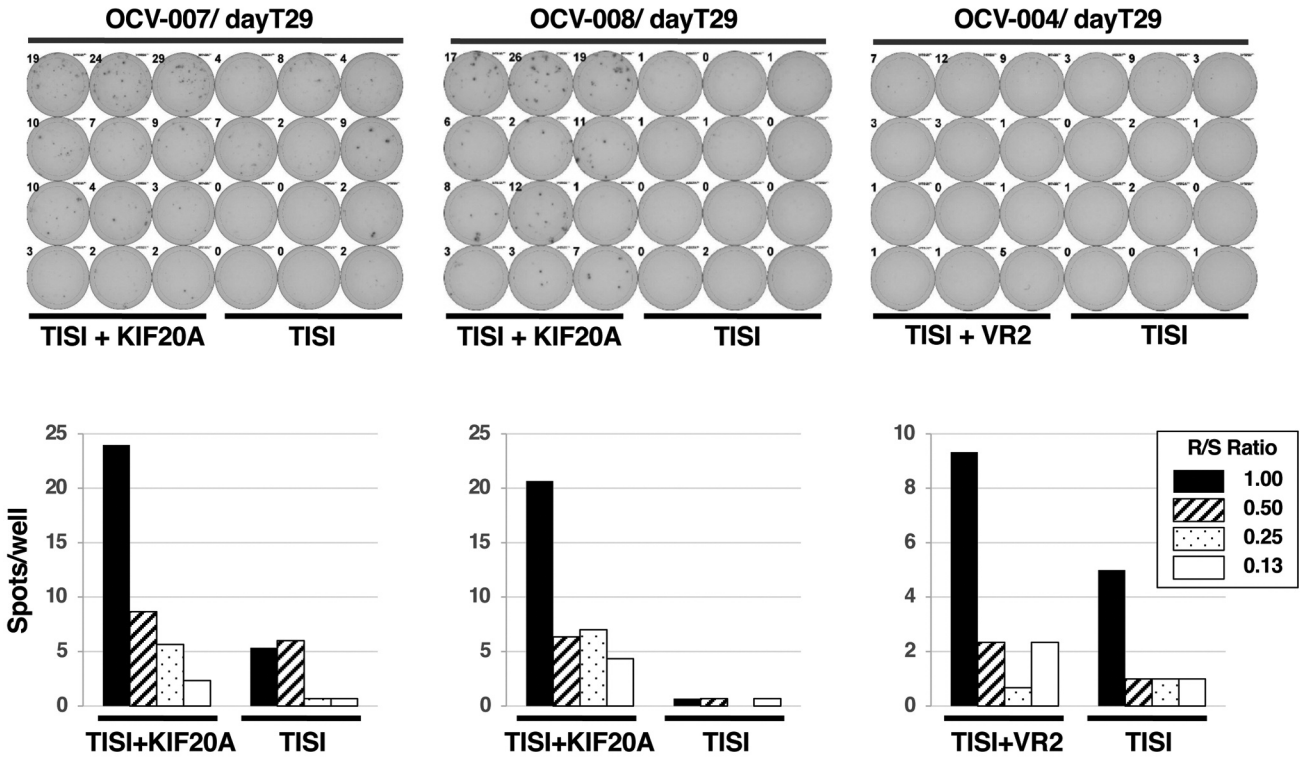


Figure 2. *Vaccine-specific CD8 T-cell responses. Representative results of the ELISPOT assay (day T29) for OCV-007, -008, and -004. "TISI (lymphoblast cells positive for HLA-A*24:02) + KIF20A" and "TISI+VR2" indicate stimulators with KIF20A and VR2 peptide pulses, respectively. "TISI" indicates stimulators alone, without a peptide pulse. R/S Ratio: responder/stimulator Ratio. All ELISPOT assays were performed in triplicate wells. The immune response is represented as positive or negative, the definition of which is detailed in "Materials and Methods" section. VR2, VEGFR2.*

no significant difference in the overall survival comparison of the HLA-A * 24:02-matched group and -unmatched group. In contrast, a significant difference was observed when comparing PFS, which is a secondary endpoint, between the HLA-A*24:02–matched and -unmatched groups (HLA-A*24:02+: MST 56 days, 95%CI=34.0-169.0; HLA-A*24:02-: MST 410.5 days, 95%CI=288.0-533.0, Log–rank test, $p$=0.0494). The best overall effect of tumour regression, another secondary endpoint, was PR in one case (16.7%), SD in two cases (33.3%), and PD in three cases (50%). The HLA-A*24:02-matched group included SD in one case and PD in three cases, and the HLA-A*24:02–unmatched group included PR and SD in one case each. Changes in serum tumour markers did not significantly differ from baseline at all time points, but some cases exhibited clear changes, as illustrated by the corresponding images (Figure 1a-c). OCV-005 achieved PR after 16 vaccinations, and her PR state persisted for 1 year. This clinical response was associated with a significant reduction in CA19-9 (Figure 1c). After 1 month of vaccination, CA19-9 was reduced to normal levels, and was maintained until withdrawal of vaccination.

*Vaccine-specific CD8 T-cell responses and survival*. The CTL responses to the three peptide vaccines were as follows: HLA-A*24:02–matched group: OCV-101 25%, OCV-105 50%; HLA-A*24:02–unmatched group: elpamotide 50%, OCV-101 50%, OCV-105 50%. ELISPOT assay revealed that four of six patients (two HLA-A*24:02–matched, two HLA-A*24:02–unmatched) exhibited vaccine-specific T cell responses to more than one of three antigens (67%). Representative results of the ELISPOT assay for cases, OCV-007, -008, and -004 are shown in Figure 2. Results of pre-vaccination and post-vaccination are compared to demonstrate the significant vaccine-induced immune response against KIF20A (007, 008) and VEGFR2 (004). Immune and clinical responses are summarised in Table III. Kaplan–Meier estimates of OS and PFS in patients treated with OCV-C01, comparing ELISPOT-positive (n=4, blue line) and -negative (n=2, red line) groups, are shown in Figure 3. Log–rank tests demonstrated that vaccine-specific T-cell responses contributed significantly to both OS (ELISPOT +: MST 566 days, ELISPOT–: MST 113 days, 95%CI=108.0-118.0, $p$=0.0177) and PFS (ELISPOT+:

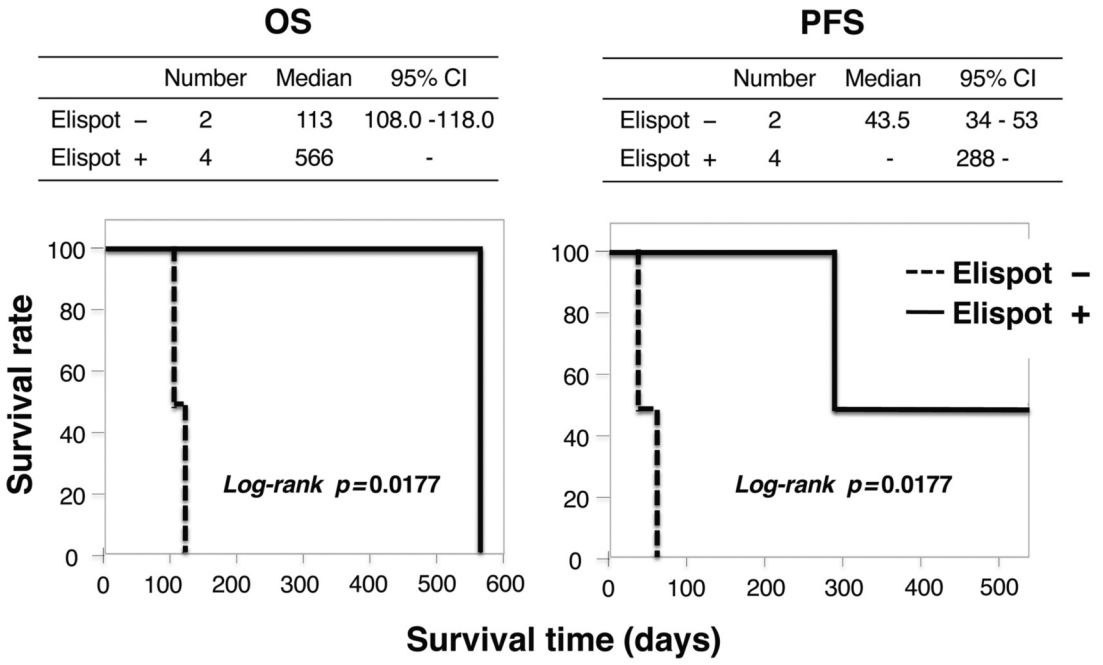


Figure 3. *Comparison of survival between ELISPOT-positive and -negative cases. Kaplan–Meier estimates of progression-free survival (PFS) and overall survival (OS) in patients treated with OCV-C01, comparing ELISPOT-positive (+) (n=4, solid line) and -negative (–) (n=2, dotted line) groups. A log–rank test between positive and negative ELISPOT findings revealed that vaccine-specific T-cell responses were associated with significantly longer OS and PFS (p=0.0177).*

Table III. *Immune responses and clinical responses.*

| Patient | Days after vaccination and CTL responses | | | | | | | | | | | | IC | HLA-A* | BOR | OS | PFS |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | dayT1 | | | dayT29 | | | dayT57 | | | dayT180 | | | | | | | |
| | VR2 | VR1 | KIF | VR2 | VR1 | KIF | VR2 | VR1 | KIF | VR2 | VR1 | KIF | KIF | | | | |
| OCV-003 | – | – | – | – | – | – | | | | | | | | 24:02 26:02 | PD | 108 | 34 |
| OCV-004 | – | – | – | + | – | – | – | + | – | – | + | – | – | 02:06 31:01 | SD | 566 | 288 |
| OCV-005 | – | – | – | – | – | – | – | – | + | – | – | – | | 02:06 26:02 | PR | 540 | 533 |
| OCV-006 | – | – | – | – | – | – | – | – | – | | | | + | 24:02 26:01 | PD | 118 | 53 |
| OCV-007 | – | – | – | – | – | + | – | + | + | | | | + | 24:02 26:01 | PD | 429 | 59 |
| OCV-008 | – | – | – | – | – | + | | | | | | | | 24:02 33:03 | SD | 365 | 169 |

CTL, Cytotoxic T lymphocytes; VR2, VEGFR2; VR1, VEGFR1; KIF, KIF20A; IC, immunochemistry; BOR, best overall response, OS, overall survival; PFS, progression free survival; PD, progressive disease; SD, stable disease; PR, partial response.

MST–, 95%CI=288–, ELISPOT–: MST 43.5 days, 95%CI=34-53, *p*=0.0177).

*Expression of KIF20A*. We retrospectively examined the expression of KIF20A in cases in which formalin-fixed tissue specimens were obtained at the time of diagnosis. Pathological analyses by IHC staining confirmed the expression of KIF20A in two of three patients for whom samples were available (67%) (Table III). KIF20A expression was confirmed in the patient in which CTLs for

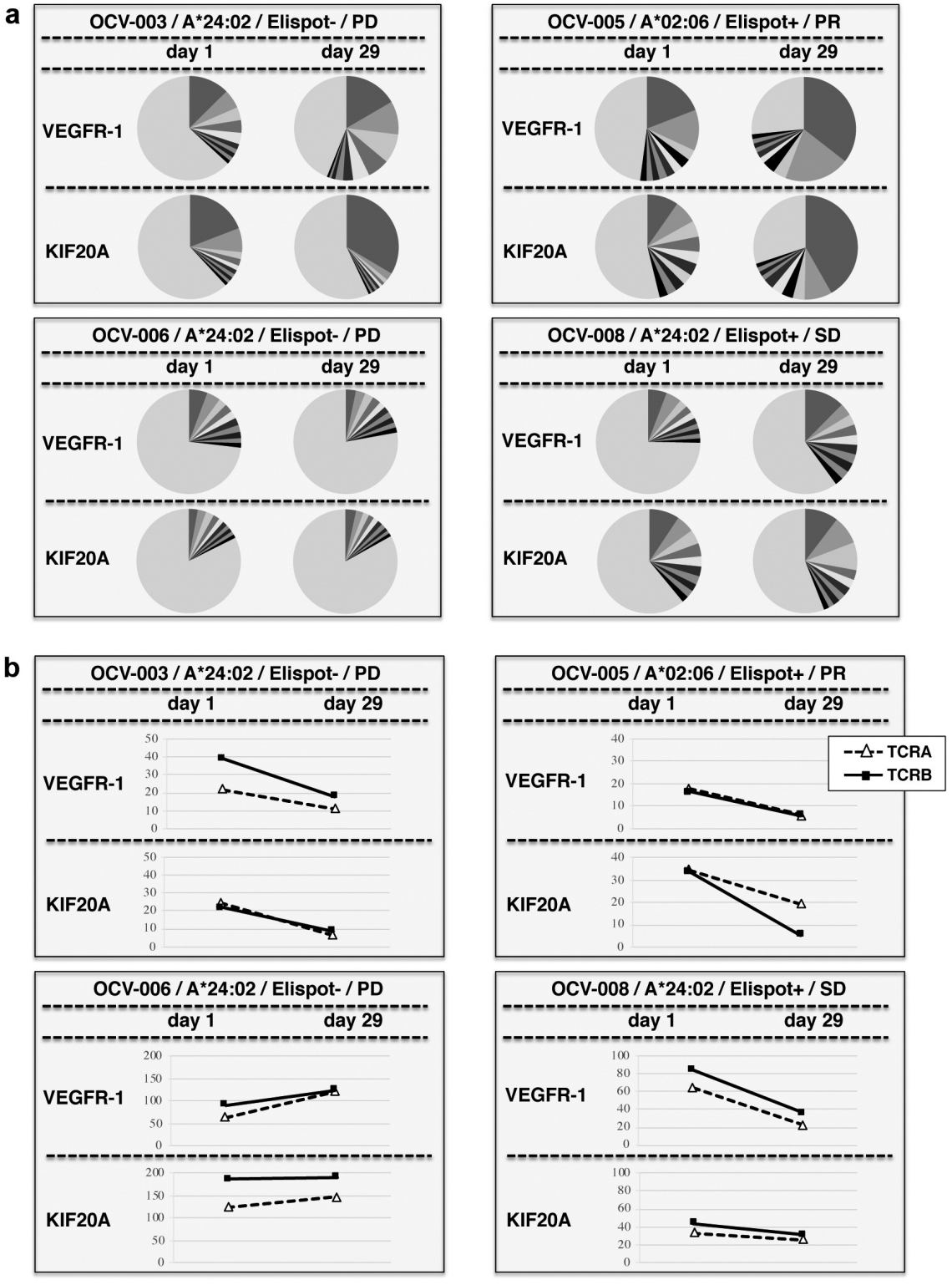


Figure 4. *TCR repertoire of vaccine-induced CD8 T cells. (a) TCRβ repertoire in ELISPOT-positive and -negative cases. We used bulk CD8+ T cells stimulated with the indicated peptides, as shown in the ELISPOT assay, to amplify TCRα,β chain cDNA and finally analyse the DNA sequence. Each pie chart colour indicates the T-cell population that expressed the same clonotypic TCRβ. The same color in different cases does not mean the same clone. Grey zone denotes the repertoire obtained from only a single T-cell clone. (b) Change in Diversity index of both TCRα and β of Vx-induced CD8 T cells. Dotted lines indicate TCRα; solid lines indicate TCRβ.*

KIF20A were induced (OCV-007). On the other hand, no CTLs for KIF20A were induced in the other KIF20A expression–positive patient (OCV-006). The KIF20A-negative patient exhibited no CTLs for KIF20A (OCV-004).

*TCR repertoire of vaccine-induced CD8 T cells*. In cases for which consent for gene analysis was obtained, we performed gene analysis of the TCR of peripheral blood-derived CD8 T cells following IVS culture, and then used a pie chart and diversity index to compare the clonality of the TCR repertoire between ELISPOT-positive and -negative cases (Figure 4a, b). In the responder cases, we observed clonally expanded populations in which T cells expressed the same clonotypic TCR.

## Discussion

BTC is a relatively rare disease. In addition, because multiple clinical trials were underway in Japan at the same time, the number of patients registered during our trial period was small, and the study was discontinued; notably however, this was not due to a safety issue but due to insufficient number of cases. Although this study was a single-group study, it was designed to compare the clinical effects of HLA-matched and -unmatched groups to obtain a proof-of-concept for this vaccine by opening HLA after the trial. In fact, vaccine-specific T-cell responses were observed in two of four HLA-A*24:02–matched group patients. On the other hand, based on the ELISPOT assay and TCR analysis, vaccine-specific T-cell responses were also induced in two cases in the HLA-A*24:02–unmatched group. It must be emphasised that these two cases exhibited prolonged survival with a clear tumour regression effect (BOR=PR and SD). Previous work has shown that the immune response by cross-reactivity provides antitumour effects even if the patient does not possess the target HLA (16). Recently, multiple clinical trials of cancer vaccines using neoantigen have been conducted, and their effectiveness is being confirmed (17-20), and attempts are ongoing to develop shared neoantigens that appear frequently in particular HLAs and use them as off-the-shelf immunotherapies (21). As described above, this study suggests that it is inappropriate to use the HLA-unmatched group as a control group in the development of vaccines. Trials of pancreatic cancer using KIF20A with the same design did not show a significant difference in survival between the HLA-matched and -unmatched groups (22). However, we cannot rule out the possibility that the HLA-unmatched group contains cross-reactive CTLs. Future cancer vaccine development should be compared between the vaccinated and non-vaccinated groups in the HLA-matched population. In a study by Aruga *et al*., CTLs for KIF20A were induced in eight of nine cases (88%) (5). In this study, CTLs were induced in three of six patients (50%) for either KIF20A or VEGF, but CTLs were induced simultaneously for both KIF20A and VEGF in only one HLA-matched patient. Theoretically, CTLs must be induced in both VEGF and tumour-specific antigens at the same time in order to obtain a synergistic effect. Therefore, the development of a vaccine that can efficiently induce CTLs on two antigens, including VEGF and a tumour-specific antigen, represents a priority for future work. Alternatively, it may be worth considering combining a cancer vaccine against a tumour-specific antigen with an anti-VEGF antibody.

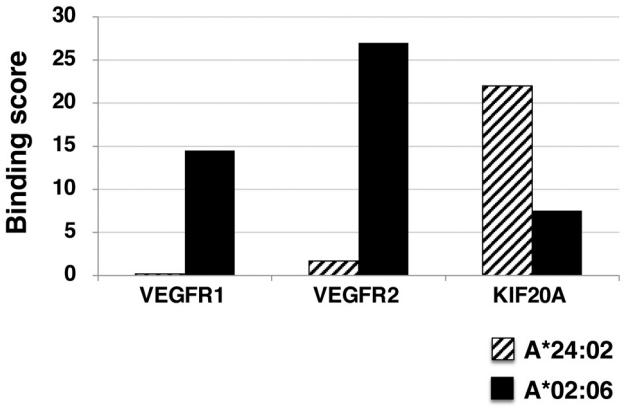


Figure 5. *Comparison of MHC-I binding predictions between A*24:02 and A*02:06. Binding scores of the peptide sequences of VEGFR1, VEGFR2, and KIF20A used in this study were calculated using IEBD Analysis Resource MHC-I Binding Predictions (http://tools.immuneepitope.org/mhci/). Striped bars indicate HLA-A*24:02; filled bars indicate HLA-A*02:06.*

Given the high prevalence of BTC in Asia and the lack of effective second-line treatments, it would be worthwhile to develop a cancer vaccine for patients carrying the alleles in cases that showed clear clinical responses in this study. We note that both of the HLA-unmatched patients had HLA-A*02:06. Considering the binding score of HLA-A*02:06 for OCV-C01, HLA-A*02:06 may be a suitable allele for OCV-C01 (Figure 5). In Japan, the HLA-A*02:06 allele has a prevalence of around 10%, which is the second highest frequency after HLA-A*24:02 (23). To develop these results, we prepared a stimulator carrying HLA*02:06 and are planning to perform a CTL assay. HLA-A*02:06 constitutes one cluster in Asia, including Japan (24). If the OCV-C01 used in this study is shown to be presented *via* HLA-A*02:06, it would be worth continuing development as an option for the treatment of BTC.

## Conflicts of Interest

K.T. reports receiving research grants from Neopharma Japan, Co., Ltd., Neoprecision therapeutics Co., Ltd. and Shinnihonseiyaku Co., Ltd. K.T. has stocks in Neoprecision therapeutics Co., Ltd. and in

Shinnihonseiyaku Co., Ltd. No potential conflicts of interest were disclosed by the other authors. S.Y. and T.H. were employees of OncoTherapy Science, Inc. and are employees of Cancer Precision Medicine, Inc..

## Authors' Contributions

M.M. conducted the experiments, prepared figures, and participated in study design and manuscript writing. M.M., T.T., K.Y., Y.H. and H.O. provided medical care for the patients who participated in the clinical trial. S.Y and T.H conducted the immunological analyses. J.K conducted the statistical analyses. Y.N. supported the clinical trial. K.T. contributed to the designs of the study and writing of the manuscript, and provided advice.

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