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Antibody responses against NY-ESO-1 and HER2 antigens in patients vaccinated with combinations of cholesteryl pullulan (CHP)-NY-ESO-1 and CHP-HER2 with OK-432

Masatoshi Aoki[a,b,c,1,2], Shugo Ueda[d,1], Hiroyoshi Nishikawa[b], Shigehisa Kitano[a], Michiko Hirayama[b], Hiroaki Ikeda[b], Hideki Toyoda[e], Kyosuke Tanaka[e], Michiyuki Kanai[d], Arimichi Takabayashi[d], Hiroshi Imai[f], Taizo Shiraishi[f], Eiichi Sato[g], Hisashi Wada[h], Eiichi Nakayama[i], Yoshiyuki Takei[c], Naoyuki Katayama[a], Hiroshi Shiku[b], Shinichi Kageyama[a,b,*]

[a] Department of Haematology and Oncology, Mie University Graduate School of Medicine, 2-174, Edobashi, Tsu, Mie 514-8507, Japan
[b] Departments of Cancer Vaccine & Immuno-Gene Therapy, Mie University Graduate School of Medicine, Mie, Japan
[c] Department of Gastroenterology, Mie University Graduate School of Medicine, Mie, Japan
[d] Department of Gastroenterological Surgery and Oncology, Kitano Hospital, Tazuke Kofukai Medical Research Institute, Osaka, Japan
[e] Department of Endoscopic Medicine, Mie University Graduate School of Medicine, Mie, Japan
[f] Department of Pathology, Mie University Graduate School of Medicine, Mie, Japan
[g] Department of Pathology, Tokyo Medical University, Tokyo, Japan
[h] Department of Surgery, Osaka University Graduate School of Medicine, Osaka, Japan
[i] Department of Immunology, Okayama University Graduate School of Medicine, Okayama, Japan

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ABSTRACT

Combination vaccines of the NY-ESO-1 protein complexed with cholesteryl pullulan (CHP), CHP-NY-ESO-1, and the truncated 146HER2 protein with CHP, CHP-HER2, were subcutaneously administered with the immuno-adjuvant OK-432 to eight esophageal cancer patients. Vaccination was well-tolerated. NY-ESO-1- and HER2-specific antibody responses were analyzed using the patients' sera and samples from previous single CHP-NY-ESO-1 or CHP-HER2 vaccine trial. The responses to NY-ESO-1 in the combination vaccine study were comparable to the single vaccine. For responses to HER2, there were fewer antibody responses in the combination vaccines. Although there were marked individual variations in the antibody responses to the NY-ESO-1 and HER2 antigens, the reaction patterns to these antigens were comparable within each patient. Antibodies to OK-432 were not augmented. Protein cancer vaccines targeting multiple antigens are feasible.

1. Introduction

Complexes of cholesteryl pullulan (CHP) nanoparticles that contain a tumour antigen are a new type of cancer vaccine with a novel antigen delivery system for both the MHC class I and class II pathways [1–3]. Previous clinical studies using CHP-HER2 and CHP-NY-ESO-1 vaccines have shown that each of these vaccines could be administered repeatedly without serious adverse effects and both vaccines induced antigen-specific $CD8^+$ and $CD4^+$ T cell immunity as well as humoral immunity [4–7]. In a CHP-NY-ESO-1 vaccine study, tumour regressions were observed in two esophageal cancer patients [6]. These findings have encouraged us to design a cancer vaccine that is more immunogenic and broadly applicable to cancer patients.

As tumour cells often express multiple tumour-specific antigens that can be recognized by T cells [8–10], a cancer vaccine targeting multiple antigens could be more efficient to simultaneously induce multiple immune responses.

NY-ESO-1 antigen is expressed in approximately 20–30% of esophageal cancers [8,11,12]. Although there has been no comprehensive report on HER2 expression in esophageal cancers, HER2 expression has been described in previous case reports [13–17]. As CHP-NY-ESO-1 and CHP-HER2 were safely administered as a single vaccine, combination vaccines targeting these two antigens would be feasible and might be efficacious in a broader cancer patient population. OK-432 has been reported to stimulate toll-like receptor (TLR)-4 and to activate antigen-presenting cells [18]. Because OK-432 was used safely with the CHP-HER2 vaccine [5],

* Corresponding author at: Departments of Cancer Vaccine & Immuno-Gene Therapy, Mie University Graduate School of Medicine, 2-174, Edobashi, Tsu, Mie 514-8507, Japan. Tel.: +81 59 231 5187; fax: +81 59 231 5276.
E-mail addresses: aoki@kaiseihp.com (M. Aoki), kageyama@clin.medic.mie-u.ac.jp (S. Kageyama).
[1] The first two authors contributed equally to this study.
[2] Tel.: +81 59 231 5016; fax: +81 59 231 5200.

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we have designed and administered CHP-HER2 and CHP-NY-ESO-1 combination vaccines with the immuno-adjuvant OK-432 to therapy-refractory esophageal cancer patients. We aimed to evaluate the safety of these combination vaccines and to analyze the humoral immune responses by co-analyzing the responses in previous CHP-NY-ESO-1 or CHP-HER2 single vaccine studies.

2. Material and methods

2.1. Preparation of NY-ESO-1 and 146HER2 proteins complexed with cholesteryl pullulan (CHP) for combination vaccines

Recombinant NY-ESO-1 and 146HER2 proteins for clinical use were prepared, and the complex consisting of CHP and the NY-ESO-1 protein, and CHP and the HER2 complex were formulated as described previously [4,19]. All processes were performed following cGMP conditions. The safety of the vaccine materials was assessed using animal models, and their biochemical and biological stabilities were examined regularly in our laboratories.

2.2. Clinical trial

A clinical trial of the combination vaccine consisting of the CHP-NY-ESO-1 and CHP-HER2 complexes was designed to evaluate their safety and the immune responses to the NY-ESO-1 and HER2 antigens. Patients were included in this study if they had therapy-refractory advanced or metastatic esophageal cancers that expressed NY-ESO-1 and/or HER2. NY-ESO-1 expression was assessed by RT-PCR using a specific primer [11] or by immunohistochemistry with the monoclonal antibody, E978 [20]. A cancer was considered HER2-positive if the examined section scored $\geq$1 by immunohistochemistry (HercepTest, Dako Corporation, Carpinteria, CA). The CHP-NY-ESO-1 and CHP-HER2 vaccines were injected subcutaneously for a total of 6 cycles with an interval of two weeks. Patients who showed no severe adverse effects after 6 cycles were allowed to continue vaccination. The injection dose for CHP-NY-ESO-1 was 100 μg of NY-ESO-1 protein, and the dose for CHP-HER2 was 300 μg of 146HER2 protein. A 0.02 mg dose of the immune-modulator OK-432 (Chugai Pharmaceuticals, Tokyo, Japan) was mixed with each vaccine solution before injection, and the two vaccines, CHP-NY-ESO-1/OK-432 and CHP-HER2/OK-432, were administered separately at the same lymph node region.

Toxicity was evaluated according to the National Cancer Institute Common Terminology Criteria for Adverse Events ver.3.0 (NCI-CTCAE ver.3.0) [21]. Clinical responses were assessed according to Response Evaluation Criteria in Solid Tumours (RECIST) after the sixth cycle of vaccination [22].

The trial was approved by the Human Ethics Committees of Mie University and Kitano Hospital, and it was conducted in full conformity with the current revision of the Declaration of Helsinki. Written informed consent was obtained from each patient enrolled in the trial. The clinical trial was registered as NCT00291473 of ClinicalTrial. gov, and 000001081 of UMIN Clinical Trial Registry.

2.3. Serum samples

To analyze antigen-specific antibody responses for the present combination trial, sera were collected before vaccination and two weeks after each vaccination.

To co-analyze the responses in the two previous studies, in which the CHP-NY-ESO-1 and CHP-HER2 vaccine were each given as a single vaccine [5,7], serum samples were collected before and up to 6 cycles after vaccination. Samples from 5 NY-ESO-1-positive esophageal cancers, N-1, N-2, N-3, N-4, and N-5, were available from the previous CHP-NY-ESO-1 single study [7], which included four males and one female with an age range of 52–72 years (median 57 years). They all received CHP-NY-ESO-1 vaccine alone without any adjuvants. Eight samples were available from the previous CHP-HER2 single study, including four breast cancers and four miscellaneous cancers, H-1 H-2, H-3, H-4, H-5, H-6, H-7, and H-8 [5]. All of these tumour samples were HER2-positive. These patients included four males and four females who ranged in age from 40 to 62 years, with a median age of 55 years. In the trial, CHP-HER2 was given as a single agent for the first four cycles. After the fifth cycle, GM-CSF or OK-432 was added as an immuno-adjuvant [4,5]. H-1 H-2, H-3, and H-4 received GM-CSF, and H-5, H-6, H-7 and H-8 were given OK-432. All sera were stored at −80 °C until analysis.

2.4. Antibody responses to the NY-ESO-1, HER2 antigens and OK-432

Specific antibodies in the sera were measured by ELISA as described previously [23]. Briefly, recombinant proteins and overlapping peptides of NY-ESO-1 or HER2 were absorbed onto immunoplates (442404; Nunc, Roskilde, Denmark) at a concentration of 10 ng/50 μL/well at 4 °C. The collected serum samples were diluted from 1:400 to 1:12,800. After washing and blocking the plate, the sera were added and incubated for 10 h. After washing, goat anti-human IgG (H + L chain)-, (MBL, Nagoya, Japan) IgG_1-, IgG_2-, IgG_3- or IgG_4-peroxidase (The Binding Site, San Diego, CA) was added. After adding the TMB substrate (Pierce, Rockford, IL), the plate was read with a Microplate Reader (model 550; Bio-Rad, Hercules, CA). An optical density $(OD)_{450}$ absorption value of at least 0.182 for NY-ESO-1 and at least 0.5 for HER2 was considered a positive reaction for a serum dilution of 1:400. For antibody-positive patients at baseline, their antibody titers were judged as "enhancement" if they changed by four-time dilutions or more.

To measure antibodies to OK-432, 10 ng/50 μL/well Picibanil™ (Chugai Pharmaceutical, Tokyo, Japan) was absorbed onto the immunoplates (442404; Nunc, Roskilde, Denmark) at 4 °C. The collected serum samples were added to the plate and assessed as described above.

2.5. Generation of recombinant proteins of NY-ESO-1 and 146HER2 and overlapping peptides

The recombinant NY-ESO-1 protein used for assays was produced using NY-ESO-1 cDNA (nt 68–607, GenBank accession number AJ275977) that was cloned into the SphI-SalI sites of the pQE30 vector (QIAGEN, Hilden, Germany). The N-His-tagged NY-ESO-1 protein was expressed in M15 *E. coli* cells and purified by nickel-ion affinity chromatography under denaturing conditions. The recombinant 146HER2 protein was prepared using HER2 cDNA. Truncated HER2 cDNA encoding amino acids 1–146 was PCR-amplified and inserted into pET15b (Novagen, Madison, WI). *E. coli* strain JM109 was transformed with the resulting plasmid and then induced with IPTG to produce the 6His-tagged truncated 146HER2 protein. The recombinant 146HER2 protein was stably produced by *E. coli* (Asahi Glass, Tokyo, Japan).

The following series of 12 overlapping NY-ESO-1 25-mer peptides, which spanned the protein and overlapped by 11 amino acids, was synthesized: 1–25, 15–39, 29–53, 43–67, 57–81, 71–95, 85–109, 99–123, 113–137, 127–151, 141–165, and 151–175. Fourteen overlapping NY-ESO-1 20-mer peptides spanning the protein were also synthesized: 1–20, 11–30, 21–40, 31–50, 41–60, 51–70, 61–80, 81–100, 91–110, 101–120, 111–130, 131–150, 151–170, and 161–180. A 25-mer peptide, 119–143, and a 22-mer peptide, 139–160, were also used. These peptides were synthesized using standard solid-phase methods based on N-(9-fluorenyl)-methoxycarbonyl (Fmoc) chemistry on a Multiple