

ARTICLE

A phase 1 study of a heterologous prime-boost vaccination involving a truncated HER2 sequence in patients with HER2-expressing breast cancer

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A phase 1 clinical trial was conducted to assess the safety, tolerability, and preliminary efficacy of a heterologous prime-boost strategy involving plasmid DNA (pHM-GM-CSF, expressing truncated human epidermal growth factor receptor 2 (HER2) and granulocyte macrophage colony-stimulation factor (GM-CSF) as a bicistronic message) and an adenoviral vector (Ad-HM, containing the same modified HER2 sequence only), in patients with stage III–IV metastatic breast cancer expressing HER2. Nine eligible subjects were divided into three cohorts based on the dosages (2, 4, and 8 mg/patient/visit) of pHM-GM-CSF used as the primer, which was intramuscularly injected three times at weeks 0, 2, and 4. It was followed by a single injection of Ad-HM (3×10^9 virus particles), used as a booster, at week 6. During the 6-month follow-up period, adverse events (AEs), pharmacokinetics and pharmacodynamics, and HER2-specific cellular and humoral immune responses were evaluated. Seven cases of minor grade 1 toxicities in four of nine subjects and no serious drug-related AEs were reported. HER2-specific cell-mediated or humoral immunity was produced in all (100%) or three subjects (33%), respectively. One subject showed a partial response, and seven subjects had stable diseases. However, there were no differences in clinical tumor response and HER2-specific immune responses among the cohorts. These results showed that intramuscular injections of pHM-GM-CSF and Ad-HM were well tolerated and safe.

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INTRODUCTION

HER2, a 185-kDa transmembrane receptor protein, is one of the tumor-associated antigens that is overexpressed in various types of tumors like breast, gastric, prostate, liver, bladder, and ovarian cancers.^{1–4} Overexpression of HER2 in human cancer correlates with poor prognosis and the severity of the disease.⁵ For example, patients with breast cancers overexpressing HER2 have been reported to have an aggressive form of the disease with more rapid progression and shortened survival time.⁶

Trastuzumab is indicated for HER2-positive patients, defined as patients diagnosed with immunohistochemistry (IHC) 3+ or IHC 2+ with fluorescence *in situ* hybridization (FISH)/silver *in situ* hybridization (SISH) positive. Although trastuzumab monotherapy has been reported to inhibit tumor growth and extend progression-free survival, only 15–20% of breast cancer patients overexpressing HER2 are known to respond to this antibody treatment.⁷ One approach to overcoming this limitation is by inducing cytotoxic T-lymphocyte response to HER2. A peptide vaccine containing the HER2 region interacting with a specific human leukocyte antigen type has been shown to induce a specific cytotoxic T-lymphocyte response and increase the disease-free survival rate.⁸ The downside of this approach, however, is

that it is restricted to a partial human leukocyte antigen type and cannot induce humoral immunity.

To overcome the limitations of the antibody and peptide combination strategy, the possibility of using DNA-based therapeutic cancer vaccines has been explored in various clinical trials, which were aimed at inducing both humoral and cellular immunities without being restricted by the human leukocyte antigen type. The most extensively studied strategy include the use of plasmid DNA with or without an adenoviral (Ad) vector designed to express the coding region of particular tumor antigens. In the context of a homologous prime-boost approach, a plasmid DNA vaccination alone was used; it is simple, safe, and can be used repeatedly. The magnitude of the immune induction, however, is generally low for desired anticancer therapeutic effects. The use of an adenoviral vector alone can elicit strong immunity, but undesirable immune responses to viral proteins present in the vector may be induced, and repeated injections are known to increase antibodies to these viral proteins rather than generating immune responses specific to target antigens.⁹ Safety and productivity of viral vector are important factors for repeat treatment. In that respect, adenoviral vector is advantageous as safety of adenoviral vector are identified by many clinical trials, and production titer is higher than other viral vectors.¹⁰

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The combinatorial use of DNA and an adenoviral vector, called a heterologous prime-boost approach, has been explored to solve the problems associated with the single use of the respective delivery systems. In this approach, a DNA vaccine is used as a primer, while a recombinant Ad vector is employed as a booster. The advantage of the heterologous prime-boost strategy is that a higher immune response to the target antigen can be induced, while nonspecific immunity against viral proteins is minimized. The enhancement of immunity to the target antigen can increase the number of antigen-specific T cells and can provide selective enrichment of high-avidity T cells, ultimately leading to increased efficacy.¹¹

Researchers have previously demonstrated that the plasmid DNA (pHM-GM-CSF) expressing both a truncated form of HER2 (HM) and granulocyte macrophage colony-stimulation factor (GM-CSF) as a bicistronic message in a mouse model could induce immune responses against HER2 and consequently, antitumor effects.¹² When a heterologous prime-boost vaccination scheme involving a plasmid (pHM-GM-CSF) and an adenoviral vector (Ad-HM) encoding only HM was applied to nonhuman primates, similar observations were made regarding safety as well as immune induction.¹³ As adjuvant, GM-CSF was selected among interleukin (IL)-12, IL-15, IL-18, osteopontin (Eta)-1, and Fms-like tyrosine kinase-3 ligand (Flt-3L) because it induced higher HER2-specific cellular immune response and reduced the tumor sizes in tumor-bearing murine.¹²

The aim of this phase 1 dose-escalation study was to evaluate the safety, tolerability, and HER2-specific immunological activities of an intramuscular heterologous prime-boost therapeutic vaccination involving pHM-GM-CSF and Ad-HM in patients with HER2-expressing breast cancer and to determine the appropriate dose.

RESULTS

Study design and population

This study was a phase 1, open-label, dose-escalation and uncontrolled single-center study designed to assess the safety, tolerability,

and immunological activities of VM206RY, a heterologous prime-boost vaccination, using a plasmid DNA (pHM-GM-CSF) and an adenoviral vector (Ad-HM), in patients with HER2-expressing breast cancer (including 1+, 2+, and 3+ as measured by IHC). The primary objective of this study was the assessments of safety, tolerability, and the identification of maximal tolerated dose of the heterologous prime-boost vaccination program. HER2-specific cellular and humoral immune responses and clinical tumor response in accordance with the Response Evaluation Criteria in Solid Tumors (RECIST) criteria 1.1 were evaluated as preliminary efficacy variables. Subjects were followed up for 24 weeks.

Sixteen subjects with breast cancer who provided informed consent form were screened for eligibility. Nine eligible subjects were subsequently assigned to cohorts 1, 2, or 3 (three subjects per cohort). As shown in Figure 1, nine subjects were treated with three different doses of pHM-GM-CSF (6, 12, and 24 mg for cohorts 1, 2, and 3, respectively) and a single dose of Ad-HM (3×10^9 virus particles (VP)). Each subject received pHM-GM-CSF intramuscularly on weeks 0, 2, and 4, followed by one Ad-HM injection 2 weeks later (on week 6). The dose-escalation decision and permission to administer at a higher dose were made by the data safety monitoring board, which independently evaluated the occurrence of adverse drug reactions and dose-limiting toxicity of the last subject of each cohort at 8 weeks post-injection.

Among the nine subjects, three were withdrawn after a safety evaluation at 8 weeks post-injection, and six subjects completed the study. One subject in cohort 1 died as a result of chemotherapy-related sepsis at week 16. Two subjects in cohort 2 voluntarily withdrew from the study at weeks 10 and 16. Subject characteristics at study entry time are presented in Table 1. The mean age was 47.2 years, with a range of 36–65 years. All the subjects were in stage IV, with metastatic tumors in bone, brain, liver, or lymph nodes.

As shown in Table 1, the HER2 expression level was determined by IHC and FISH or by SISH. Seven subjects were diagnosed as 3+ or 2+ by IHC and positive for FISH or SISH, and two were diagnosed as 1+ or 2+ by IHC and negative for FISH or SISH. All subjects with high

Cohort	Primer DNA (mg/visit)			Booster Adenoviral vector (vp)			Injection									
	2	4	8	2	4	8	0	2	4	6	8	10	12	16	20	24
1	2	2	2	2	2	2										
2	4	4	4	4	4	4										
3	8	8	8	8	8	8										
Safety evaluation	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Serum protein level of HER2	✓	✓	✓	✓	✓	✓								✓		✓
Plasma protein level of GM-CSF	✓					✓										
Adenoviral antibodies to GM-CSF	✓					✓										
Antibodies to HER2	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
CTL response to HER2	✓					✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

Figure 1 A dose-escalation study of pHM-GM-CSF. Subjects were immunized with 2, 4, or 8 mg of pHM-GM-CSF at weeks 0, 2, and 4 and then boosted with 3×10^9 VP of Ad-HM at week 6. All adverse events were monitored at every visit and evaluated during the study period. The samples for measuring pharmacokinetics (the presence of HER2 and GM-CSF proteins and Ad-HM vector), GM-CSF autoantibody, and immunological response to HER2 (humoral and cell-mediated immunities) were collected periodically during the study.

Table 1 Subject characteristics

		Cohort			Total
		1	2	3	
Number of subjects		3	3	3	9
Mean age (SD)		44.7 (10.0)	42.7 (7.7)	54.3 (12.9)	47.2 (10.5)
ECOG performance status, n (%)	0	1 (33.3)	0 (0.0)	1 (33.3)	2 (22.2)
	1	2 (66.7)	3 (100.0)	2 (66.7)	7 (77.8)
HER2 expression, n (%)	Low	0 (0.0)	0 (0.0)	2 (66.7)	2 (22.2)
	High	3 (100)	3 (100)	1 (33.3)	7 (77.8)
Metastatic site, n (%)	Bone	1 (33.3)	2 (66.7)	1 (33.3)	4 (44.4)
	Brain	2 (66.7)	1 (33.3)	0 (0.0)	3 (33.3)
	Distant LNs	1 (33.3)	1 (33.3)	2 (66.7)	4 (44.4)
	Liver	2 (66.7)	2 (66.7)	1 (33.3)	5 (55.6)
	Local regional LNs	0 (0.0)	1 (33.3)	0 (0.0)	1 (11.1)
	Lung	3 (100.0)	3 (100.0)	2 (66.7)	8 (88.9)
	Other	1 (33.3)	0 (0.0)	0 (0.0)	1 (11.1)
	Skin/soft tissue	1 (33.3)	1 (33.3)	1 (33.3)	3 (33.3)

ECOG, Eastern Cooperative Oncology Group; LN, lymph node.

Table 2 Cancer treatment before study entry and after vaccination

Class of agent, n (%)	Cohort			Total
	1	2	3	
Before study entry				
Chemotherapy				
Antimetabolites ^a	2 (66.7)	0 (0.0)	1 (33.3)	3 (33.3)
Antimicrotubules ^b	1 (33.3)	2 (66.7)	2 (66.7)	5 (55.6)
Target agent ^c	2 (66.7)	2 (66.7)	1 (33.3)	5 (55.6)
Chemotherapy combination ^d	3 (100)	3 (100)	3 (100)	9 (100)
Hormone therapy ^e	2 (66.7)	1 (33.3)	1 (33.3)	4 (44.4)
Supportive therapy ^f	1 (33.3)	1 (33.3)	1 (33.3)	3 (33.3)
After vaccination				
Chemotherapy				
Cytotoxic antibiotics ^g	1 (33.3)	0 (0.0)	0 (0.0)	1 (11.1)
Antimicrotubules ^h	1 (33.3)	0 (0.0)	1 (33.3)	2 (22.2)
Chemotherapy combination ⁱ	2 (66.7)	2 (66.7)	1 (33.3)	5 (55.6)
Hormone therapy ^j	1 (33.3)	1 (33.3)	0 (0.0)	2 (22.2)
Supportive therapy ^k	2 (66.7)	1 (33.3)	1 (33.3)	4 (44.4)

^aTwo subjects in cohort 1 (capecitabine) and one subject in cohort 3 (gemcitabine) had received antimetabolite chemotherapeutic agents before study entry.^bOne subject in cohort 1 (paclitaxel), two subjects in cohort 2 (paclitaxel), and two subjects in cohort 3 (docetaxel or paclitaxel) had received microtubule-targeted chemotherapeutic agent.^cTwo subjects in cohort 1, two subjects in cohort 2, and one subject in cohort 3 had received trastuzumab as HER2-targeted agent.^dNine subjects had been treated with combination regimens; 5-FU+cyclophosphamide+methotrexate, cyclophosphamide +doxorubicin, carboplatin+paclitaxel, capecitabine+vinorelbine, gemcitabine+vinorelbine, 5-FU+doxorubicin+cyclophosphamide, trastuzumab+paclitaxel, vinorelbine+gemcitabine, capecitabine+lapatinib, pazopanib+lapatinib, doxorubicin+docetaxel, gemcitabine+cisplatin.^eHormone therapy: tamoxifen, exemestane, and letrozole.^fSupportive therapy: goserelin, zoledronic acid, and pamidronate.^gOne subject in cohort 1 (mitomycin and doxorubicin) had received cytotoxic antibiotics agents after vaccination.^hOne subject in cohort 1 (vinblastine) and one subject in cohort 3 (paclitaxel) had received microtubule-targeted chemotherapeutic agent.ⁱFive subjects had been treated with combination regimens; 5-FU+cyclophosphamide+methotrexate, mitomycin+vinblastine, doxorubicin+cyclophosphamide, and gemcitabine+vinorelbine.^jOne subject in cohort 1 and 2 had received letrozole hormone therapy.^kSupportive therapy: methylprednisolone, pamidronate, and zoledronic acid.

Table 3 Adverse events

	Cohort					
	1		2		3	
	Number of subjects	Number of events	Number of subjects	Number of events	Number of subjects	Number of events
Adverse drug reaction						
Number (%) of subjects	2 (66.7)	5	1 (33.3)	1	1 (33.3)	1
Musculoskeletal and connective tissue disorder						
Myalgia	1 (33.3)	3	0 (0.0)	0	1 (33.3)	1
General disorders and administration site conditions						
Pyrexia	0 (0.0)	0	1 (33.3)	1	0 (0.0)	0
Fatigue	1 (33.3)	1	0 (0.0)	0	0 (0.0)	0
Skin and subcutaneous tissue disorder						
Blister	1 (33.3)	1	0 (0.0)	0	0 (0.0)	0
Serious treatment-emergent adverse event						
Number (%) of subjects	2 (66.7)	5	3 (100.0)	8	1 (33.3)	1
Respiratory, thoracic, and mediastinal disorders						
Pleural effusion	0 (0.0)	0	2 (66.7)	4	0 (0.0)	0
Dyspnea	1 (33.3)	2	0 (0.0)	0	0 (0.0)	0
Pneumothorax	1 (33.3)	1	0 (0.0)	0	0 (0.0)	0
Infections and infestations						
Pneumonia	1 (33.3)	1	0 (0.0)	0	0 (0.0)	0
Wound infection	0 (0.0)	0	1 (33.3)	1	0 (0.0)	0
Nervous system disorders						
Dizziness	0 (0.0)	0	1 (33.3)	1	0 (0.0)	0
Headache	1 (33.3)	1	0 (0.0)	0	0 (0.0)	0
Blood and lymphatic system disorders						
Febrile neutropenia	0 (0.0)	0	1 (33.3)	1	0 (0.0)	0
General disorders and administration site conditions						
Chest pain	0 (0.0)	0	1 (33.3)	1	0 (0.0)	0
Hepatobiliary disorders						
Bile duct obstruction	0 (0.0)	0	0 (0.0)	0	1 (33.3)	1

expression level of HER2 had previously received standard therapy, including trastuzumab, lapatinib, taxane, and capecitabine prior to enrollment. Subjects with low expression level of Her2 also received combined or single standard chemotherapy involving anthracycline, taxane, gemcitabine, capecitabine, vinorelbine, and cyclophosphamide prior to enrollment. After the vaccination program, three subjects in cohort 1, two subjects in cohort 2, and two subjects in cohort 3 with symptomatic progressions were administered with appropriate chemotherapies. One subject in cohort 2 received hormone therapy prior to enrollment until week 4 (Table 2).

Safety and tolerability

The heterologous vaccination of the pHM-GM-CSF prime and the Ad-HM boost were well tolerated at all dose levels, and a maximal tolerated dose could not be determined in this study. During the

treatment and follow-up period, none of the subjects experienced a decline in left ventricle ejection fraction below 50% and cardiac-related adverse events (AEs), often observed in subjects using trastuzumab. As shown in Table 3, four subjects experienced seven minor adverse drug reactions, such as myalgia, pyrexia, fatigue, blister, etc. The most commonly reported AEs were nausea, decreased appetite, cough, dyspnea, pleural effusion, insomnia, and pyrexia. Fourteen serious AEs of grade 2 or more that were observed in six subjects were considered to be unrelated to the study drug. Of the 14 serious AEs, one subject death was reported during the follow-up phase due to pneumonia sepsis after chemotherapy.

Clinical tumor response

All the subjects had at least one measurable lesion and were therefore eligible according to RECIST criteria 1.1. As summarized in

Table 4 Clinical tumor response

Cohort	1	2	3	Total
Response rate, <i>n</i> (%)	0 (0.0)	0 (0.0)	1 (33.3)	1 (11.1)
Exact 95% CI	(0.0, 70.8)	(0.0, 70.8)	(0.8, 90.6)	(0.3, 48.2)
Disease control rate, <i>n</i> (%)	3 (100.0)	2 (66.7)	3 (100.0)	8 (88.9)
Exact 95% CI	(29.2, 100.0)	(9.4, 99.2)	(29.2, 100.0)	(51.8, 99.7)
Best overall response, <i>n</i> (%)				
Complete response	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Partial response	0 (0.0)	0 (0.0)	1 (33.3)	1 (11.1)
Stable disease	3 (100.0)	2 (66.7)	2 (66.7)	7 (77.8)
Progressive disease	0 (0.0)	1 (33.3)	0 (0.0)	1 (11.1)

Table 4, there was one subject (11.1%) with partial response rate and eight subjects (88.9%) with the controlled disease. One subject in cohort 3 (11.1%) achieved partial response (PR) at week 16 and was maintained until the last visit (24 weeks). Seven subjects (77.8%) achieved stable disease (SD) in cohorts 1, 2, and 3. One (11.1%) subject was assessed as progressive disease (PD) at week 8 in cohort 2. There were no differences in clinical tumor response among the cohorts.

Pharmacokinetics and pharmacodynamics

The pharmacokinetics and pharmacodynamics of the two reagents used in this heterologous prime-boost vaccination program were studied by measuring the levels of the GM-CSF and HER2 proteins, the adenoviral vector (Ad-HM), and the autoantibodies to GM-CSF at pre- and postvaccination times in all subjects. Plasma GM-CSF remained stable over the range of 0–0.1 pg/ml without any peak until 2 weeks after pHM-GM-CSF administration for all subjects. Antibodies to GM-CSF were not detected in plasma at week 2 after the Ad-HM vaccination. In whole blood from eight subjects, the Ad-HM vector was less than lower limit of the quantitation at week 2 after the Ad-HM administration. In one subject, a residual amount of Ad-HM vector DNA was transiently detected over the range of 92.07–102.65 copies per μ g of genomic DNA (gDNA) until 4 weeks after Ad-HM injection but was undetectable at week 7 after the Ad-HM injection. The subsequent molecular analysis revealed that this DNA was from Ad-HM, not from replication-competent adenovirus. The median baseline serum HER2 level of enrolled subjects was 227.9 ng/ml (range: 20.1–438.6 ng/ml) for subjects with HER2-overexpressing cancer, whereas it was 10.8 ng/ml (range: 9.5–12.6 ng/ml) for subjects with low HER2-expressing cancer. In subjects with HER2-overexpressing cancer, two subjects showed a decline in the serum protein level of HER2 by 10–50% in cohort 1, whereas the level increased in five subjects by 1.3- to 19.4-fold. In two subjects with low HER2-expressing cancer, the serum HER2 level dropped below the normal range of 15 ng/ml.

Specific cell-mediated immunity to HER2

To evaluate the production of specific cell-mediated immunity (CMI) to HER2 after the pHM-GM-CSF primed and Ad-HM boosted vaccination, the HER2-specific T-cell response was analyzed by an interferon (IFN)- γ enzyme-linked immunospot (ELISPOT) assay using peripheral blood mononuclear cells from each subject. As shown in

Table 5, specific CMI to HER2 was induced in all subjects during the study period and persisted until week 20 in two subjects. Among the subjects, three showed specific CMI after the DNA vaccine only, while the rest produced CMI after the adenoviral vector injection. A \geq 6-fold higher level of specific CMI relative to respective controls was observed in four subjects (two subjects in cohort 1, one subject in cohort 2, and one subject in cohort 3). However, there was no correlation between the DNA vaccine dosage and the level of HER2-specific CMI.

Specific antibody formation to HER2

The level of HER2-specific antibody formation in subjects' plasma was analyzed using cell-based enzyme-linked immunosorbent assay (ELISA) and fluorescence activated cell sorting (FACS). When the former method was used, three subjects produced a detectable level of the HER2 antibody (Table 6). One subject in cohort 1 had \sim 2.5 μ g/ml of the HER2 antibody at week 10. One subject in cohort 2 produced the HER2 antibody at weeks 4, 10, and 16. One subject in cohort 3, who received the highest dosage of the DNA vaccine (24 mg), generated positive responses at weeks 12 and 24. Overall, no dose-dependent response was observed. When FACS analysis was employed, no positive response was observed.

DISCUSSION

In this phase 1 clinical study involving patients with HER2-expressing breast cancer, we showed that vaccination with pHM-GM-CSF priming followed by Ad-HM boosting was well tolerated and that HER2-specific immunogenicity could be induced.

Seven cases of grade 1 drug-related reactions, such as fatigue and myalgia, were observed in four of nine subjects, while no serious drug-related AEs were reported. There was no evidence of a clinically significant reduction in left ventricular function, a cardiotoxicity often observed during the use of trastuzumab.¹⁴ Abnormal levels of GM-CSF proteins from the expression of plasmid DNA and antibodies due to autoimmune reaction against this cytokine were not detected.

Eight of nine subjects showed no sign of DNA from the adenoviral vector genome after adenoviral boosting. In one subject, however, the viral DNA was transiently detected 2 weeks after the injection but disappeared 5 weeks later at week 7. Subsequent analysis demonstrated that this viral DNA originated from Ad-HM, not replication-competent adenovirus.

Table 5 Induction of HER2-specific cell-mediated immunity

Cohort	Allocation no		Follow-up (weeks)								
			0	6	8	10	12	16	20	24	
1	101	Unpulsed	3	20	8	17	37	42	n/a	n/a	
		Stimulation	3	95	44	37	73	37	n/a	n/a	
		+ or –	–	+	+	+	–	–	n/a	n/a	
	102	Unpulsed	34	3	3	0	1	n/a	n/a	n/a	
		Stimulation	57	32	13	0	0	n/a	n/a	n/a	
		+ or –	–	+	+	–	–	n/a	n/a	n/a	
	103	Unpulsed	0	0	18	1	0	5	0	0	
		Stimulation	0	0	21	3	8	11	9	0	
		+ or –	–	–	–	–	+	+	+	–	
2	201	Unpulsed	8	20	3	158	3	49	38	71	
		Stimulation	14	18	5	81	8	25	20	103	
		+ or –	–	–	–	–	+	–	–	–	
	202	Unpulsed	4	2	4	n/a	n/a	n/a	n/a	n/a	
		Stimulation	7	8	4	n/a	n/a	n/a	n/a	n/a	
		+ or –	–	+	–	n/a	n/a	n/a	n/a	n/a	
	203	Unpulsed	2	19	12	2	7	n/a	n/a	n/a	
		Stimulation	2	11	12	15	14	n/a	n/a	n/a	
		+ or –	–	–	–	+	+	n/a	n/a	n/a	
	3	301	Unpulsed	15	23	9	30	1	22	9	4
			Stimulation	18	20	22	48	1	8	11	7
			+ or –	–	–	+	–	–	–	–	–
302		Unpulsed	31	14	10	3	51	13	8	29	
		Stimulation	19	21	16	9	33	38	30	31	
		+ or –	–	–	–	+	–	+	+	–	
303		Unpulsed	70	46	7	31	3	9	50	43	
		Stimulation	37	10	82	17	5	6	49	64	
		+ or –	–	–	+	–	–	–	–	–	

CMI against HER2 was measured with an IFN- γ ELISPOT assay using fresh peripheral blood mononuclear cells from subjects at baseline and weeks 6, 8, 10, 12, 16, 20, and 24. Specific CMI to HER2 was induced with more than sixfold higher level compared to an unpulsed control in four subjects (102, 103, 203, and 303). Cells unpulsed with peptides served as negative control (mock). A positive response was defined as ≥ 5 spot-forming cells per well and a twofold or greater increase compared with the negative control. n/a, not available due to withdrawal.

In three subjects, the HER2 protein level in serum increased after Ad-HM boosting. There are two possible explanations. First, HER2 proteins expressed by the Ad-HM injected in local muscles may have been released into the bloodstream. Second, the extracellular domain of HER2 may have been released from HER2-overexpressing malignant tumor cells, based on the reports by Lin *et al.*¹⁵ and Zabrecky *et al.*¹⁶ We believe the second explanation is more likely. The three subjects showing an increased level of HER2 had progressive cancers; one subject had a life-threatening tumor, while two subjects showed PD at weeks 8 and 16. Indeed, other subjects maintaining disease stability showed no significant changes in the HER2 protein level in serum following Ad-HM administration. Additional techniques, such as western blotting, are needed to determine whether HER2 proteins in serum are expressed by advanced tumors or result from the vaccine.

In this study, eight subjects achieved disease control with one confirmed PR and seven having SD. Although it is difficult to precisely evaluate the clinical response of VM206RY injections because these subjects have also been treated with different chemo- and/or hormone therapies after the vaccination program, VM206RY is deemed to have produced highly positive results as vaccinated subjects with late-stage breast cancer had failed in multiple lines of prior chemotherapies (Tables 2 and 4).

In one subject, the HER2 protein level in serum decreased to normal levels (from 43 to 12 ng/ml) after being treated with doxorubicin + cyclophosphamide and letrozole between weeks 8 and 24. PR was observed due to a decrease in the tumor target lymph node lesion. It is interesting to note that in this subject there was more than a sixfold increase in CMI compared to the control. It is plausible that the use of VM206RY contributed to this improvement because

Table 6 Level of the HER2-specific antibody ($\mu\text{g/ml}$) in three subjects

Subjects	Weeks									
	0	2	4	6	8	10	12	16	20	24
101	0	0	0	0	0	2.56	0	0	n/a	n/a
201	0	0	17.26	0	0	2.67	0	3.12	0	0
303	3.83	1.95	0	0	0	0	4.75	2.9	3.1	4.14

n/a, not available due to withdrawal.

no anticancer effects had previously been observed in this subject when treated heavily with multiple lines of anticancer therapies including the use of trastuzumab, cyclophosphamide, doxorubicin, gemcitabine, and 5-FU prior to this study.

Anticancer vaccination strategies similar to VM206RY have been employed in other studies. Data from a phase 1 trial involving a DNA vaccine-primed and adenoviral vector-boosted HER2/carcinoembryonic antigen showed that strong cellular and humoral immunities against the lymphotoxin beta protein used as a genetic adjuvant were induced, while no specific immune responses to the actual tumor antigens, HER2 and carcinoembryonic antigen, were detected.¹⁷ In another study, tumor antigen-specific CMI was observed in 13 of 29 subjects (45%) with prostate-specific antigen-expressing prostate cancer. Five of 29 subjects showed greater than a sixfold increase in CMI compared to baseline, which contributed to improving overall survival, according to the authors.¹⁸

Cell-mediated immunity to HER2 was used as one of the efficacy parameters in this study. CMI was detectable in all subjects, while a sixfold higher level of CMI compared to the respective control was observed in four subjects (44%). The presence of HER2 antibodies was measured as an additional parameter, which was observed in three subjects (33%). One of the three subjects showed HER2 antibody levels (17 $\mu\text{g/ml}$) similar to a minimum serum trough concentration (C_{\min}) of trastuzumab.¹⁹

This phase 1 study provides several important guidelines for future clinical studies. First, the maximal tolerated dose of VM206RY has yet to be determined by other dose-escalation study in the future. Second, the injection and dosing schemes have to be modified to maintain immune responses for longer period and to confirm the correlation between the VM206RY dosage and the level of HER2-specific immune response. We previously observed in the canine model that repeated DNA injection after an adenoviral vector boosting could help to re-induce the immune responses and be maintained for 5 months. Also, in the study by Walter *et al.*, a relatively large amount of adenoviral vector (1×10^{10} or 10^{11} particle units) could result in maintaining cellular and humoral immune response against HIV-1 for 24 weeks,²⁰ while in the study by Conry *et al.*, repetitive dosing of DNA vaccine induced antigen-specific antibodies with protective antibody level,²¹ suggesting that the injection scheme could highly influence the outcome. Third, the combinatorial use of VM206RY vaccination program and conventional anticancer therapies needs to be investigated. Certain chemotherapy involving gemcitabine and cyclophosphamide was reported to diminish the immune suppressor function of regulatory T cells.²² In addition, we also observed that combining injections of VM206RY and chemotherapy increased anticancer and immunomodulating effects in the preclinical study.²³

In summary, our data show that intramuscular injections of pHM-GM-CSF (24 mg, ~ 0.4 mg/kg) and Ad-HM 3×10^9 VP ($\sim 5 \times 10^7$ VP/kg) were well tolerated and safe. This heterologous prime-boost vaccination strategy quite effectively induced CMI and, to a lesser extent, antibody responses. To our knowledge, this is the first study demonstrating the induction of HER2-specific cellular and humoral responses in subjects with metastatic breast cancer expressing HER2. Although the clinical outcomes of VM206RY could not be precisely determined because of a small size of this study and the use of other anticancer therapies, it is thought that this vaccination program might have exerted highly positive effects as enrolled subjects had previously in multiple lines of chemotherapies. Since the induction of both humoral and cellular response has been observed in a relatively larger number of patients, a larger-scale phase 2 study is warranted to more extensively investigate the safety and efficacy of this heterologous priming and boosting strategy.

MATERIALS AND METHODS

Study materials and preparation of the studied products

VM206RY is an intramuscular heterologous prime-boost therapeutic vaccine consisting of a plasmid (pHM-GM-CSF) expressing the extracellular and transmembrane domains (HM) of human HER2 minus the intracellular region together with GM-CSF genes as a bicistronic message in the pCK backbone^{12,24} and adenoviral vector (Ad-HM) expressing HM in the commercially available type 5 recombinant adenovirus backbone (TaKaRa Bio, Japan) as previously described.²⁵ pHM-GM-CSF was manufactured by Cobra Biologics (Keele, UK) and lyophilized by Formatech (Andover, MA), whereas Ad-HM was made by SAFC Pharma (Madison, WI), in compliance with the good manufacturing practice standards of the US FDA. pHM-GM-CSF was supplied in a sterile glass vial containing 2.2 mg of lyophilized plasmid DNA (pDNA) and stored at 2–8 °C until injection. Lyophilized pDNA was reconstituted with 2.2 ml of water (1 mg/ml) and resuspended for 5 minutes at room temperature for injection. Ad-HM was supplied in a sterile glass vial containing 8.5×10^{10} VP/ml and was stored at 20–80 °C until injection. Before injection, frozen Ad-HM was completely thawed at room temperature, after which 0.5 ml of Ad-HM was diluted with 11.3 ml of saline (3×10^9 VP/ml). The VM206RY was injected into brachial and deltoid muscle.

Subject eligibility

This study (<http://www.clinicaltrials.gov> registration number: NCT01895491) was conducted in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice, approved by the Korea Food and Drug Administration and by the Institutional Review Board of the Asan Medical Center (Seoul, Korea). Patients older than 20 years of age who had HER2-expressing metastatic breast cancer, measurable lesion(s) in the breast or metastasized sites, an Eastern Cooperative Oncology Group score ≤ 3 at the time of study entry, and who completed standard therapies and progressed beyond trastuzumab and lapatinib at least 4 weeks prior to enrollment met the inclusion criteria. Additionally, subjects were required to have the following normal laboratory findings in hematological screening tests: white blood cell count $\geq 3000/\mu\text{l}$; platelet $> 100,000/\mu\text{l}$; hematocrit ≥ 30 ; total bilirubin $< 1.5\times$; aspartate aminotransferase, and/or alanine aminotransferase $< 2.5\times$ upper limit of normal; and creatinine $< 1.5\times$ upper limit of normal. Exclusion criteria included uncontrolled cardiac disease, coronary artery disease, autoimmune disease, and immune deficiency disease. In addition, patients were excluded if they received a cumulative dose of doxorubicin > 360 mg/m² or epirubicin > 720 mg/m² by the time of study entry and had clinically abnormal values of antinuclear Ab, anti-double-stranded DNA, and C3 in a screening test.

Concomitant medications and therapies

Subjects were prohibited chemo-, corticosteroids, immune-suppressive, and radiation therapies during 8 weeks after the first injection. Particularly, anthracyclines such as doxorubicin and epirubicin were prohibited during the 24 weeks follow-up period. Also, trastuzumab and herbal medicines that might affect immune system were prohibited during the follow-up period. However, biophosphonate and hormone therapy started prior to enrollment were permitted continuous treatment over the study period.

Safety

All AEs were monitored and recorded at every visit and were evaluated according to CTCAE (Common Terminology Criteria for Adverse Events) version 4.0 issued by the NCI (National Cancer Institute) during the study period. Dose-limiting toxicity was defined as AEs higher than grade 3 based on the NCI-CTCAE version 4.0 with respect to their relationship with the study drug and injection procedure until 2 weeks after the final injection. The maximal tolerated dose was defined as the highest dose evaluated for which less than one-third of the participants experienced dose-limiting toxicity.

Clinical tumor response

Clinical tumor response was analyzed by computed tomography based on the RECIST version 1.1. This included PD, SD, PR, and CR at baseline during weeks 4, 8, 16, and 24. The response rate was defined as the percentage of subjects with advanced or metastatic cancer who have achieved CR or PR. The disease control rate was defined as the percentage of subject with CR, PR, or SD to a therapeutic intervention in clinical trials of anticancer agents.

Pharmacokinetics and pharmacodynamics analysis

Changes in GM-CSF and HER2 levels were evaluated pre- and post-injection (Figure 1). The levels of plasma GM-CSF and serum HER2 were determined by an ELISA using a commercially available ELISA kit (R&D system), according to the manufacturer's instructions. To demonstrate no persistence after Ad-HM treatment in humans (i.e., to prove the disappearance of Ad-HM in subjects after Ad-HM injection), gDNA was obtained from whole blood using a commercially available kit (Qiagen), according to the manufacturer's instructions (Figure 1). Ad-HM vector levels in whole blood were measured by the quantitative polymerase chain reaction (qPCR) developed and validated by ViroMed (Seoul, Korea). If any Ad-HM vector remained in whole blood after treatment, a 103-bp fragment would be amplified from gDNA samples by qPCR using specific primers to the poly A and HM sequences. The lower limit of the quantitation of qPCR was 83.3 copies per 1 µg of gDNA. qPCR was performed until a confirmed negative result (less than lower limit of the quantitation) was found after treatment. GM-CSF autoantibodies in plasma were analyzed at pre- and post-injection (Figure 1) by a competitive ELISA developed by ViroMed. Briefly, the wells of MediSorp flat-bottom ELISA plates (Nunc, Denmark) were coated with a recombinant human GM-CSF protein (Humanzyme) at 4 °C for 15 hours. After washing four times with phosphate-buffered saline (PBS, containing 0.1% Tween-20) to remove unbound antigen, the wells were blocked with protein-free buffer for 4 hours at 4 °C. A polyclonal anti-GM-CSF antibody (PeproTech) serially diluted twofold (from 1,250 to 39 pg/ml), using diluted plasmas from each subject (1/5, 1/50, or 1/500, respectively) was added to the GM-CSF coated wells and incubated at 37 °C for 2 hours. After washing four times with PBS, horseradish peroxidase-conjugated streptavidin (diluted 1:1,000; BD Bioscience) was added and incubated for 30 minutes at 22 °C. After four times of extensive washing with PBS, the tetramethylbenzidine substrate was added in accordance with the manufacturer's instructions (Pierce). The standard curves of three diluted samples from each subject were graphed, and if the differences in slope precision among the three standard curves were less than 15%, the detection of GM-CSF autoantibodies was interpreted as negative.

IFN-γ ELISPOT analysis

Cell-mediated immunity against HER2 was measured using fresh peripheral blood mononuclear cells from each subject by an IFN-γ ELISPOT assay, which was modified by using commercially available kits (Mabtech, Sweden) at weeks 6, 8, 10, 12, 16, 20, and 24 (Figure 1). Peripheral blood mononuclear cells, 2.5×10^5 or 5×10^5 per well, were briefly overlaid and stimulated for 96 hours with pools of 10-mer peptides covering the entire coding sequence of HM (328 peptides, 0.5 µg final concentration). The positive immune responses inducing the stimulation were defined as when both of the following conditions were met: (i) at least five spot-forming cells per well were present and (ii) the number of spot-forming cells per well was twofold or higher compared with an unpulsed sample (negative control).²⁶ CD3 was used as a positive control.

Analysis of anti-HER2 antibody

Antibody formation against HER2 was measured using a FACS method or a cell-based ELISA as previously described, with a slight modification, at baseline and at weeks 2, 4, 6, 8, 10, 12, 16, 20, and 24 (Figure 1).^{12,27} The HER2-expressing murine colorectal carcinoma (HER2/CT26) cell line was

developed by the transduction of CT26 cells with a retroviral vector expressing the extracellular and transmembrane domains of human HER2 protein as previously published.²⁸ HER2/CT26 cells, 5×10^5 cells, were incubated with the serially diluted plasma of each subject for 1 hour at 4 °C. After washing the plate, HER2/CT26 cells were incubated with goat antihuman IgG-pentosidine (Southern Biotech) for 30 minutes at 4 °C. HER2-specific antibodies were measured using a FACS Caliber (BD Biosciences). A positive titration was defined as the greatest dilution point where the mean absorbance was more than twice that of baseline plasma. The HER2-expressing human breast carcinoma (SK-BR-3) cell line was purchased from the American Type Culture Collection (Manassas). Cell-based ELISA using SK-BR-3 cells was performed to quantify the levels of anti-HER2 antibody. This method was developed and validated by ViroMed. Plasma from each subject was briefly diluted 1:2,000 in PBS and incubated with a human IgG-coated plate for 4 hours at 37 °C to eliminate the effects of interfering factors. A total of 5×10^5 SK-BR-3 cells was seeded on collagen-coated flat-bottom 96-well plates (Iwaki, Japan) and cultured for 24 hours at 37 °C. After washing with PBS, the SK-BR-3 cell-seeded plates were incubated with plasma from each subject for 2 hours at 22 °C. The plates were then washed four times with PBS and incubated at 22 °C for 30 minutes with goat antihuman-IgG-horseradish peroxidase (1:5,000). This antibody complex was colorized using a tetramethylbenzidine substrate kit, and the level of anti-HER2 antibody was calculated in comparison to a standard curve of trastuzumab.

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