The Impact of HER2/*neu* Expression Level on Response to the E75 Vaccine: From U.S. Military Cancer Institute Clinical Trials Group Study I-01 and I-02

Linda C. Benavides,[1] Jeremy D. Gates,[1] Mark G. Carmichael,[2] Ritesh Patel,[3] Jarrod P. Holmes,[4] Matthew T. Hueman,[5] Elizabeth A. Mittendorf,[6] Dianna Craig,[4] Alexander Stojadinovic,[3] Sathibalan Ponniah,[6] and George E. Peoples[1,6]

Abstract

Purpose: HER2/*neu*, a source of immunogenic peptides, is expressed in >75% of breast cancer patients. We have conducted clinical trials with the HER2/*neu* E75 peptide vaccine in breast cancer patients with varying levels of HER2/*neu* expression. Vaccine response based on HER2/*neu* expression level was analyzed.

Experimental Design: Patients were stratified by HER2/*neu* expression. Low expressors ($n = 100$) were defined as HER2/*neu* immunohistochemistry (IHC) 1^+ to 2^+ or fluorescence *in situ* hybridization < 2.0. Overexpressors ($n = 51$) were defined as IHC 3^+ or fluorescence *in situ* hybridization ≥ 2.0. Additional analyses were done stratifying by IHC status (0-3^+). Standard clinocopathlogic factors, immunologic response (*in vivo* delayed-type hypersensitivity reactions; *ex vivo* human leukocyte antigen A2:immunoglobulin G dimer assay), and clinical responses (recurrence; mortality) were assessed.

Results: Low-expressor (control, 44; vaccinated, 56) versus overexpressor patients (control, 22; vaccinated, 29) were assessed. Low expressors, overexpressors, and most IHC-status vaccinated groups responded immunologically. Vaccinated low-expressor patients had larger maximum immunologic responses compared with overexpressor patients ($P = 0.04$), and vaccinated IHC 1^+ patients had increased long-term immune response ($P = 0.08$). More importantly, compared with controls, low-expressor patients had a mortality reduction ($P = 0.08$). The largest decrease in mortality was seen in IHC 1^+ patients ($P = 0.05$). In addition, a subset of overexpressor patients ($n = 7$) received trastuzumab before vaccination, and this combination seems safe and immunologically beneficial.

Conclusions: Most patients with various levels of HER2/*neu* expression responded immunologically and seemed to benefit from vaccination. The low expressors, specifically IHC 1^+ patients, had more robust immunologic responses and may derive the greatest clinical benefit from the E75 vaccine.

HER2/*neu* is a member of the epidermal growth factor receptor family and encodes a 185-kd tyrosine kinase receptor involved in regulating cell growth and proliferation (1, 2). Overexpression and/or amplification of the HER2/*neu* proto-oncogene is found in 25% of invasive breast cancers and is associated with more aggressive tumors and poor clinical outcome (3–5).

HER2/*neu* status is determined predominately via two methods, immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH). IHC detects expression of HER2/*neu* protein and is reported on a semiquantitative scale ranging from 0 to 3^+ (0, negative; 1^+, low expression; 2^+, intermediate expression; 3^+, overexpression; ref. 6). The FISH test detects amplification (excess copy numbers) of the HER2/*neu* gene and

Authors' Affiliations: [1]Department of Surgery, General Surgery Service, Brooke Army Medical Center, Houston, Texas; [2]Department of Medicine, Hematology and Medical Oncology Service, and [3]Department of Surgery, General Surgery Service, Walter Reed Army Medical Center, Washington, District of Columbia, [4]Joyce Murtha Breast Care Center, Windber Medical Center, Windber, Pennsylvania, [5]Department of Hematology and Medical Oncology, Naval Medical Center San Diego, San Diego, California, [6]Cancer Vaccine Development Program, U.S. Military Cancer Institute, Department of Surgery, Uniformed Services University of the Health Sciences, Bethesda, Maryland, and [7]Department of Surgical Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas

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Requests for reprints: George E. Peoples, Department of Surgery, General Surgery Service, Brooke Army Medical Center, 3851 Roger Brooke Drive, Fort Sam, Houston, TX 78234. Phone: 210-916-1117; Fax: 210-916-6658; E-mail: george.peoples@amedd.army.mil.

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Translational Relevance

This article evaluates the impact of HER2/*neu* expression level on response in phase II trials of E75 peptide with immunoadjuvant granulocyte macrophage colony-stimulating factor vaccine in disease-free, node-positive and high-risk, node-negative breast cancer patients. Patients were stratified as low expressors (n = 100), defined as HER2/*neu* 1^+ to 2^+ or fluorescence *in situ* hybridization < 2.0, or overexpressors (n = 51), defined as HER2/*neu* 3^+ or fluorescence *in situ* hybridization > 2.0. The low-expressor patients had larger immune responses and decreased mortality rates. Additional analyses were done stratifying patients according to immunohistochemistry (IHC) status, ranging from 0 to 3^+. This analysis by IHC status revealed a more robust immune response in IHC 1^+ patients, also with a decreased mortality. Overall, patients with all levels of HER2/*neu* expression, to include antigen naïve patients, responded immunologically to the E75 + granulocyte macrophage colony-stimulating factor vaccine, and the data suggests an improved benefit of vaccination in low-expressing HER2/*neu* patients, specifically IHC 1^+ tumors.

is expressed as a ratio of HER2/*neu* to chromosome 17. For many years and for most of the current trial, FISH amplification was clinically interpreted as FISH ≥ 2.0 copies (7). In 2007, the guideline was revised to define amplification as FISH ≥ 2.2 copies (6). The concurrence rates between IHC and FISH results are ~90% (8). Traditionally, FISH has been considered the gold standard because retrospective analyses reveals it to be a better predictor of trastuzumab response, and it is more objective and reproducible, albeit more expensive (6, 9, 10). However, IHC is used by most clinical laboratories currently, with FISH reserved for use in IHC 2^+ patients to determine trastuzumab qualification.

Identification and quantification of HER2/*neu* as a proto-oncogene has led to targeted immunotherapy to include trastuzumab. The latter is a recombinant humanized monoclonal antibody that binds the extracellular juxtamembrane domain of HER2/*neu* protein (11). Trastuzumab is indicated for HER2/*neu* overexpressing (IHC 3^+ or FISH ≥ 2.2) node-positive and metastatic breast cancer patients (12, 13). However, trastuzumab is not indicated for patients with low- to intermediate-HER2/*neu*-expressing tumors, which constitute >50% of breast cancer patients, because trastuzumab has shown only limited activity in these patients (14).

Another form of targeted immunotherapy currently under investigation is vaccination targeting HER2/*neu*. HER2/*neu* is a tumor-associated antigen, and peptides derived from the protein can stimulate the immune system to recognize and eliminate HER2/*neu*-expressing cancer cells (15). E75 (KIFGSLAFL, HER2/neu, 369-377) is a peptide derived from the HER2/*neu* protein's extracellular domain that is in use in clinical trials as a preventative anticancer vaccine that stimulates cytotoxic T lymphocytes (16–21). Our group has combined E75 with the immunoadjuvant granulocyte macrophage colony-stimulating factor (GM-CSF) and vaccinated immunocompetent disease-free, node-positive and high-risk, node-negative patients after completion of standard of care therapies (22).

We have previously published the results of our phase II trial (23), and in this article, we have analyzed the response to E75 vaccination based on HER2/*neu* expression levels. This includes analysis of low expressors (IHC 1^+-2^+ or FISH < 2.0) versus overexpressors (IHC 3^+ or FISH ≥ 2.0) and by IHC category (0, 1^+, 2^+, 3^+). In addition, we present initial clinical safety data and immunologic data showing a potential synergistic effect of combined trastuzumab therapy and E75 vaccination.

Materials and Methods

Patient characteristics and clinical protocols. The E75 node-positive and node-negative trials were approved by the institutional review boards and conducted at Walter Reed Army Medical Center, Washington, DC, and the Joyce Murtha Breast Care Center, Windber, PA, under investigational new drug application (BB-IND9187). All patients had histologically confirmed breast cancer and had completed a standard course of surgery, chemotherapy, and radiation (as required) before study enrollment. Patients on hormonal therapy were continued on their adjuvant hormonal regimen. After informed consent, breast cancer patients were enrolled to a stage-specific trial (node positive or node negative) and human leukocyte antigen typed because E75 binds primarily to the major histocompatibility complex class I allele *HLA-A2* (human leukocyte antigen A2) found in ~40% to 50% of the general population (24). Human leukocyte antigen A2–positive patients were vaccinated, and human leukocyte antigen A2–negative patients were observed prospectively for signs of clinical recurrence. human leukocyte antigen A3–positive patients were also vaccinated because E75 also binds to this major histocompatibility complex class I allele (an additional 15-25% of the general population; refs. 25, 26). Before vaccination, patients underwent skin testing for a panel of recall antigens (Mantoux test). Patients were considered immunocompetent if they reacted (>5 mm) to two or more antigens.

Vaccination. The E75 peptide was produced commercially using good manufacturing practices grade by NeoMPS, Inc. Peptide purity (>95%) was verified by high-performance liquid chromatography and mass spectrometry. Sterility and general safety testing was carried out by the manufacturer. Lyophilized peptide was reconstituted in 0.5 mL normal saline at 100, 500, or 1,000 μg. The peptide was mixed with immunoadjuvant GM-CSF (Berlex) in 0.5 mL normal saline. The 1.0 mL peptide-immunoadjuvant inoculation was split and administered intradermally at two sites 5 cm apart on the same extremity.

Vaccination series. The node-positive trial was designed as a two-stage safety trial with escalating doses of peptide in the initial stage and alterations of schedule in the latter stage. Details of the vaccine series have been previously published (22). Briefly, three to six patients were each assigned to receive four or six monthly injections of 100, 500, or 1,000 μg of E75 peptide (100:6, 500:4, 500:6, 1,000:4, and 1,000:6, respectively). Groups were ultimately expanded to determine and confirm optimal dosing in node-positive patients, resulting in the larger number of patients in the latter dose groups.

The node-negative trial was designed to further delineate optimal biological dose by varying the dose of GM-CSF and altering the inoculation schedule. Twelve patients with HER2/*neu*-negative (IHC 0) tumors were allowed in this trial to determine the feasibility of vaccinating a presumably antigen-naïve host. Ten patients were assigned to each dose group with constant E75 peptide dose of 500 μg assigned to receive three, four, or six monthly injections with varying GM-CSF doses (125 or 250 μg).

Toxicity. Patients were observed 1 h postvaccination for immediate hypersensitivity and returned 48 to 72 h later to have their injection sites measured, at which time they were questioned about toxicities. Toxicities were graded by the National Cancer Institute Common

Table 1. Demographics, prognostic factors, and treatment profiles of patients enrolled in E75 phase II trial by low expressors versus overexpressors (A) and HER2/*neu* expression level (B)

A

	LE control (*n* = 44)	LE vaccine (*n* = 56)	*P*	OE control (*n* = 22)	OE vaccine (*n* = 29)	*P*
Median age, y	55	56		50	52	
Range, y	31-82	27-77	0.7	32-75	37-68	0.1
Race						
White, %	86.4	89.3	0.8	72.7	86.2	0.3
Other, %	13.6	10.7	0.8	27.3	13.8	0.3
Tumor size						
T_2-T_4, %	38.6	33.9	0.7	31.8	34.5	0.9
Histologic grade						
Grade 3, %	27.2	30.4	0.8	63.6	62.1	0.9
NP, %	54.5	58.9	0.7	90.1	55.2	0.06
Hormone receptor negative, %	15.9	19.6	0.8	27.3	62.1	0.02*
Chemotherapy, %	72.7	75.0	0.8	86.4	96.6	0.3
XRT, %	84.1	75.0	0.3	72.7	75.9	NS
Hormonal therapy, %	81.8	76.8	0.6	63.6	41.4	0.2
Herceptin, %	0.2	0.2	NS	9.1	24.1	0.3

B

	0 Control (*n* = 5)	0 Vaccine (*n* = 7)	*P*	1+ Control (*n* = 15)	1+ Vaccine (*n* = 25)	*P*	2+ Control (*n* = 24)	2+ Vaccine (*n* = 26)	*P*	3+ Control (*n* = 13)	3+ Vaccine (*n* = 19)	*P*
Median age, y	50	60		54	54		50	57		49	51	
Range, y	38-74	31-74	0.4	44-82	42-71	0.4	31-75	27-77	0.2	31-74	37-62	0.2
Race												
White, %	100.0	71.4	0.5	73.3	84.0	0.4	87.5	92.3	0.7	61.5	89.5	0.1
Other, %	0.0	28.6	NS	26.7	16.0	NS	12.5	7.7	NS	40.5	10.5	NS
Tumor size												
T_2-T_4, %	40.0	14.3	0.5	66.7	28.0	0.05*	29.2	46.2	0.2	38.5	36.8	0.8
Histologic grade												
Grade 3, %	20.0	14.3	0.6	33.3	36.0	0.7	37.5	38.5	0.9	61.5	57.9	0.8
NP, %	0.0	0.0	NS	80.0	60.0	0.3	79.2	80.8	0.8	100.0	42.1	0.003*
Hormone receptor negative, %	20.0	14.3	0.6	13.3	28.0	0.4	16.7	11.5	0.9	38.5	63.2	0.2
Chemotherapy, %	80.0	42.9	0.3	80.0	76.0	0.9	87.5	96.2	0.5	92.3	94.7	0.6
XRT, %	100.0	42.9	0.08	66.7	76.0	0.7	87.5	96.2	0.5	69.2	94.7	0.1
Hormonal therapy, %	80.0	85.7	0.6	80.0	72.0	0.9	79.2	73.1	0.6	53.8	73.7	0.3
Herceptin, %	0.0	0.0	NS	0.0	0.0	NS	8.3	7.7	0.9	7.7	10.5	0.7

Abbreviations: LE, low expressor; OE, overexpressor; NP, node positive; XRT, X-ray therapy; NS, not significant.
*Statistically significant difference.

Terminology Criteria for Adverse Events v3.0. Progression from one dose group to the next occurred only if no significant toxicity occurred in the preceding dose group. Patient-specific results are reported based on maximal local and systemic toxicity occurring during the series.

Peripheral blood mononuclear cell isolation and cultures. Blood was drawn before each vaccination and at 1 (postvaccine) and 6 mos (long term) after vaccine series completion. Fifty milliliters of blood was drawn, and peripheral blood mononuclear cells were isolated. Peripheral blood mononuclear cells s were washed and resuspended in culture medium and used as a source of lymphocytes as described previously (27).

Human leukocyte antigen A2: Immunoglobulin dimer assay. The presence of $CD8^+$ E75-specific cells in freshly isolated peripheral blood mononuclear cells from patients was assessed directly by dimer assay (28). Briefly, the human leukocyte antigen A2:immunoglobulin dimer (Pharmingen) was loaded with the E75 or control peptide (E37; folate-binding protein; refs. 25–33; RIAWARTEL) by incubating 1 μg of dimer with an excess (5 μg) of peptide and 0.5 μg of β_2-microglobulin (Sigma) at 37°C overnight, then stored at 4°C until used. Peripheral blood mononuclear cells were washed and resuspended in Pharmingen Stain Buffer and added at 5×10^5 cells/100 μL/tube in 5-mL round-bottom polystyrene tubes (Becton Dickinson) and stained with the loaded dimers and antibodies. In each patient, the level of $CD8^+$ E75-specific cells was determined in response to each successive vaccination and average postinoculation levels were compared with preinoculation levels.

Delayed-type hypersensitivity. In both trials, delayed-type hypersensitivity (DTH) reaction was assessed after 100 μg of E75 peptide in 0.5 mL of normal saline (without GM-CSF) and 0.5 mL normal saline as a volume control 1 mo after completion of the vaccination series as previously described (22). The DTH reaction was measured in two dimensions at 48 to 72 h using the sensitive ballpoint-pen method, reported as the orthogonal mean, and compared with control (29). In the node-negative trial, a DTH test was done prevaccination as well. Pre-post – DTH responses were compared using unpaired two-tailed Student *t* test because node-positive patients did not have pre-DTH test done. A correlation between $CD8^+$ E75-specific cells and DTH has been previously reported (30).

Clinical recurrences. All patients were observed for signs of clinical recurrence. Disease recurrence was defined as biopsy proven or if treated for recurrence by the primary oncology team.

Statistical analysis. Recurrence rates were estimated for each treatment group using Kaplan-Meier method and compared with log-rank test. Comparison of clinical, demographic, and prognostic

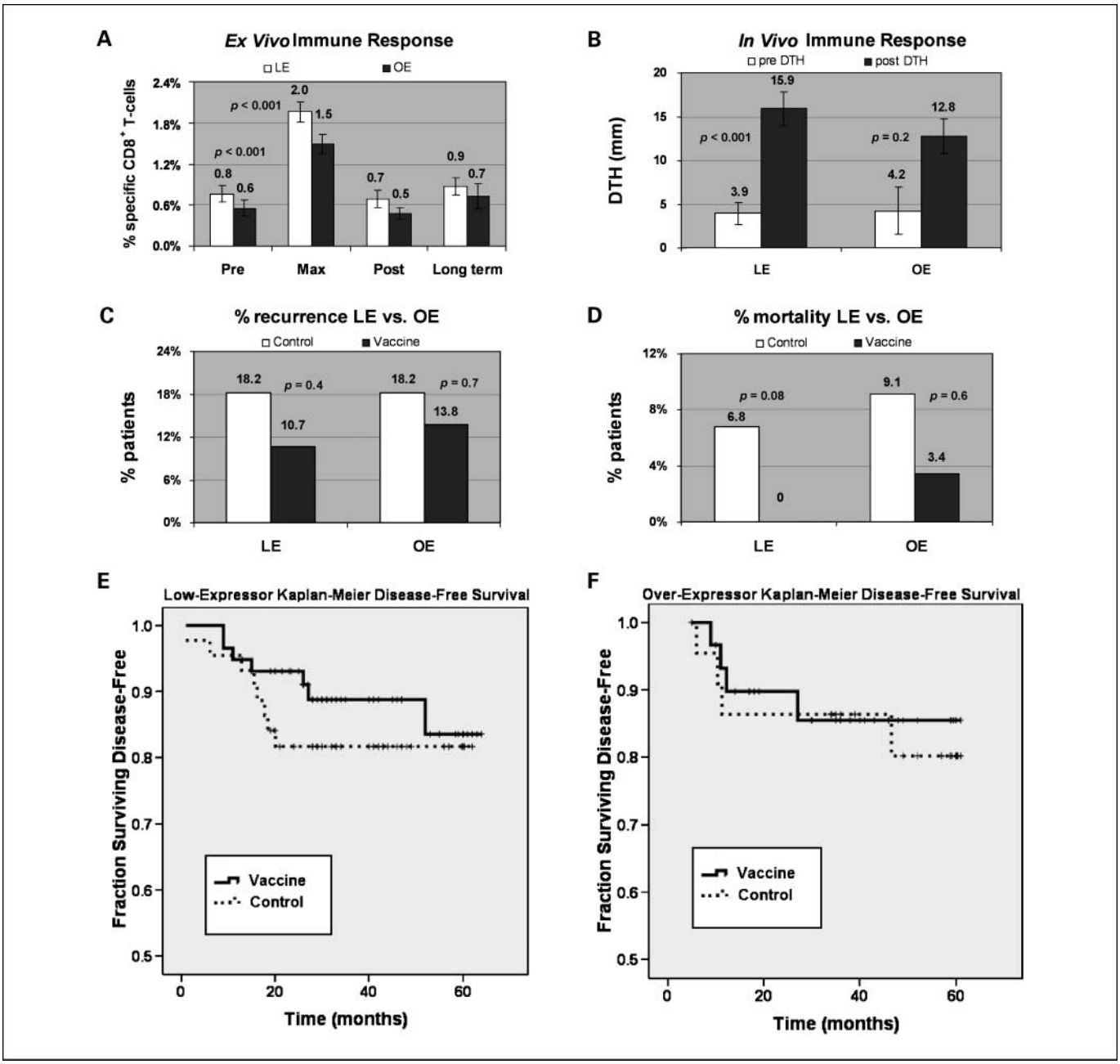

Fig. 1. Immunologic and clinical responses (recurrence and mortality rates) of patients enrolled in E75 phase II trial by HER2/*neu* low expressors versus overexpressors. *A, ex vivo* immune response (mean ± SE): all *ex vivo* pre – maximum percentage specific $CD8^+$ T cells statistically increased (low expressors, $P < 0.001$; overexpressors, $P < 0.001$); and low-expressor patients had increased maximum response compared with overexpressor patients ($P = 0.04$). *B, in vivo* immune response (mean ± SE): all *in vivo* pre-post DTHs statistically increased (low expressors; $P < 0.001$, overexpressors, $P = 0.02$). *C,* absolute recurrence rates: recurrence rates decreased in vaccinated low-expressor and overexpressor patients, albeit not statistically significant. *D,* absolute mortality rates: vaccinated low-expressor patients had a trend toward decreased mortality rates ($P = 0.08$). *E-F,* Kaplan-Meier curve for the disease-free survival of low-expressor and overexpressor patients.

parameters were by Wilcoxon, Fisher's exact test, or χ^2 as appropriate. Vaccine dimer levels and pre-post – DTH responses were calculated using paired or unpaired two-tailed Student *t* test as appropriate. $P < 0.05$ was considered statistically significant.

Results

Patients

The E75 vaccine node-positive and node-negative phase II trials enrolled 186 patients; nine withdrew, including four control patients and five vaccinated patients. No patient withdrew due to toxicity, and 177 patients completed the trial. The node-positive trial enrolled 91 patients (vaccinated, 45; control, 46), all of whom had IHC, FISH, or both tests done. The node-negative trial enrolled 86 patients (vaccinated, 51; control, 35). IHC and/or FISH data were available in 72 of these patients, including 12 patients (vaccinated, 7; control, 5) that had HER2/*neu* IHC 0 tumors. The 14 patients (vaccinated, 7; control, 7) in the node-negative trial that did not have IHC or FISH data available were excluded

from subset analysis, leaving a study population of 163 patients.

Low-Expressors versus Overexpressors Subset Analysis

Patients per HER2/neu expression category. Subset analysis was done comparing HER2/*neu* low expressors (IHC 1^+-2^+ or FISH < 2.0) versus overexpressors (IHC 3^+ or FISH ≥ 2.0). The control group had 66 patients (low expressors, 44; overexpressors, 22), and the vaccinated group had 85 patients (low expressors, 56; overexpressors, 29). Patients with IHC 0 tumors were not included in this initial analysis but are discussed later. A comparable number of control and vaccinated patients were in the low-expressor (67% versus 66%, respectively) and overexpressor groups (33% versus 34%, respectively).

Demographics, prognostic factors, and treatment profiles of low-expressor and overexpressor patients are presented in Table 1A. Among low-expressor patients, no statistical differences were noted between control and vaccinated patients. Among overexpressor patients, a greater number of vaccinated patients were hormone receptor negative than in the control group (P = 0.02; Table 1A).

Immunologic response per HER2/neu expression category. The E75 vaccine was capable of eliciting an *ex vivo* immune response in most patients, regardless of the extent of HER2/*neu* expression. Significant increases from prevaccination to maximum postvaccination E75-specific $CD8^+$ T cells were noted in both groups (low expressors, P < 0.001; overexpressors, P < 0.001). Low-expressor patients had higher maximum immune response compared with overexpressor patients (2.0 ± 0.2% versus 1.5 ± 0.1%, respectively; P = 0.04; Fig. 1A).

Low-expressor and overexpressor patients showed an *in vivo* immune response to the vaccine as measured by DTH pre- and postvaccine. Significant pre-post–DTH increases were noted in both groups of vaccinated patients (low expressors, P < 0.001; overexpressors, P = 0.02; Fig. 1B). Although the low-expressor post-DTH exceeds overexpressor post-DTH (15.9 ± 1.9 mm

Table 2. Overall and 24-mo recurrence rates and overall mortality rates with mean ± SE and median (range) by low expressors versus overexpressors (A) and HER2/*neu* expression level (B)

A

	Control LE	Vaccine LE	*P*	Control OE	Vaccine OE	*P*
Recurrence rate	18.2% (8/44)	10.7% (6/56)	0.4	18.2% (4/22)	13.8% (4/29)	0.7
Time to recurrence						
Mean ± SE	14 ± 2.3	21 ± 6.9	0.3	18 ± 10.0	15 ± 4.1	0.8
Median (range)	16 (1-20)	13 (9-52)	NS	11 (2-47)	12 (9-27)	0.7
Mortality rate	6.8% (3/44)	0.0% (0/56)	0.08	9.1% (2/22)	3.4% (1/29)	0.6
Time to death						
Mean ± SE	31 ± 6.7	N/A	N/A	20 ± 17.5	20	NS
Median (range)	37 (18-39)	N/A	N/A	20 (2-37)	20	NS
Overall follow-up						
Mean ± SE	32 ± 2.3	31 ± 2.0	0.8	41 ± 3.8	29 ± 2.9	0.01
Median (range)	31 (8-60)	28 (7-60)	0.7	40 (2-60)	28 (5-60)	0.01
Recurrence rate (24 mos)	26.7% (8/30)	12.1% (4/33)	0.2	15.0% (3/20)	15.8% (3/19)	NS
Time to recurrence						
Mean ± SE	14 ± 2.3	11 ± 1.4	0.5	8 ± 2.8	11 ± 0.9	0.6
Median (range)	16 (1-20)	10 (9-15)	0.3	10 (2-11)	11 (9-12)	0.5

B

	Control 0 (*n* = 5)	Vaccine 0 (*n* = 7)	*P*	Control 1 (*n* = 15)	Vaccine 1 (*n* = 25)	*P*	Control 2 (*n* = 24)	Vaccine 2 (*n* = 26)	*P*	Control 3 (*n* = 13)	Vaccine 3 (*n* = 19)	*P*
Recurrence rate	0.0% (0/5)	0.0% (0/7)	NS	26.7% (4/15)	8.0% (2/25)	0.2	20.8% (5/24)	15.4% (4/26)	0.7	23.1% (3/13)	15.8% (3/19)	0.7
Time to recurrence												
Mean ± SE	N/A	N/A	N/A	16 ± 1.2	12 ± 3.0	0.2	18 ± 8.0	25 ± 9.9	0.6	8 ± 2.8	11 ± 0.9	0.4
Median (range)	N/A	N/A	N/A	17 (13-18)	12 (9-15)	0.3	16 (1-47)	19 (9-52)	0.7	10 (2-11)	11 (9-12)	0.5
Mortality rate	0.0% (0/5)	0.0% (0/7)	NS	20.0% (3/15)	0.0% (0/25)	0.05	0.0% (0/24)	0.0% (0/26)	NS	15.4% (2/13)	5.3% (1/19)	0.6
Time to death												
Mean ± SE	N/A	N/A	N/A	31 ± 6.7	N/A	N/A	N/A	N/A	N/A	20 ± 17.5	20	N/A
Median (range)	N/A	N/A	N/A	37 (18-39)	N/A	N/A	N/A	N/A	N/A	20 (2-37)	20	NS
Overall follow-up												
Mean ± SE	27 ± 6.1	28 ± 4.9	0.8	32 ± 3.9	28 ± 2.8	0.8	39 ± 3.7	34 ± 3.6	0.4	39 ± 5.0	28 ± 3.4	0.06
Median (range)	18 (14-42)	26 (11-47)	NS	32 (11-60)	28 (11-60)	0.8	36 (8-60)	29 (7-60)	0.4	39 (2-60)	23 (5-60)	0.06
Recurrence rate (24 mos)	0.0% (0/2)	0.0% (0/4)	NS	36.4% (4/11)	12.5% (2/16)	0.2	21.1% (4/19)	12.5% (2/16)	0.7	25.0% (3/12)	27.3% (3/11)	NS
Time to recurrence												
Mean ± SE	N/A	N/A	N/A	16 ± 1.2	12 ± 3.0	0.2	11 ± 4.4	10 ± 1.0	0.9	8 ± 2.8	11 ± 0.9	0.4
Median (range)	N/A	N/A	N/A	17 (13-18)	12 (9-15)	0.3	11 (1-20)	10 (9-11)	NS	10 (2-11)	11 (9-12)	0.5

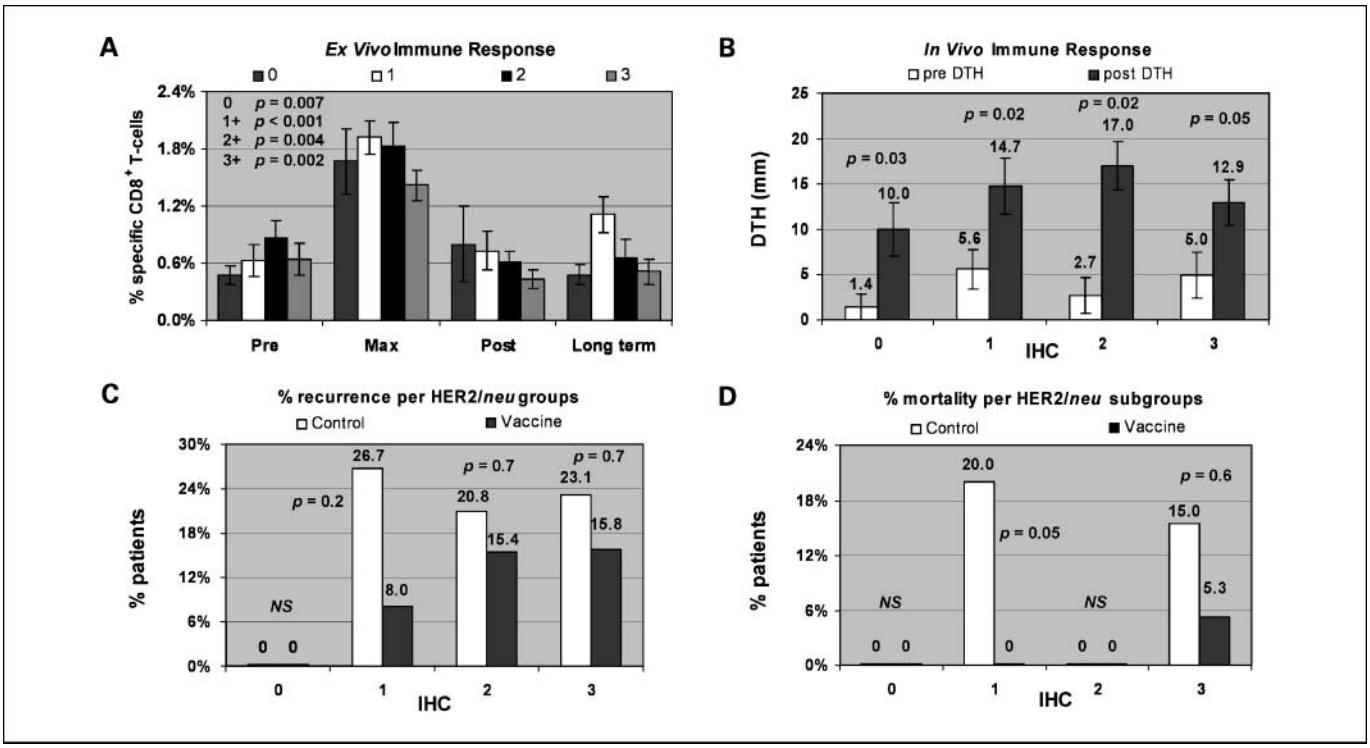

Fig. 2. Immunologic and clinical responses (recurrence and mortality rates) of patients enrolled in E75 phase II trial by HER2/*neu* IHC expression level (0, 1^+, 2^+, 3^+). *A, ex vivo* immune response (mean ± SE): all *ex vivo* pre – maximum percentage specific $CD8^+$ T cells statistically increased, whereas only HER2/*neu* 1^+ pre – long term trended toward significance ($P = 0.08$). *B, in vivo* immune response (mean ± SE): all *in vivo* pre-post DTHs statistically increased (0, $P = 0.03$; 1^+, $P = 0.02$; 2^+, $P = 0.02$; 3^+, $P = 0.05$). *C,* absolute recurrence rates: recurrence rates decreased in all vaccinated IHC levels, albeit not statistically significant. *D,* absolute mortality rates: mortality rates decreased in all vaccinated IHC levels and was statistically significant in HER2/*neu* IHC 1^+ vaccine patients ($P = 0.05$).

versus 12.8 ± 2.0, respectively), there is no significant difference between the two ($P = 0.5$). Taken together, these data suggest that the E75 vaccine is more immunologically active in low-expressor patients.

Clinical response per HER2/neu expression category. Disease recurrence and mortality are shown in Fig. 1C to F and Table 2A. All vaccinated patients (low expressors, 10.7%; overexpressors, 13.8%) had decreased recurrence rates when compared with the control patients (low expressors and overexpressors, 18.2%), but these differences were not statistically significant (low-expressor control-vaccinated, $P = 0.4$; overexpressor control-vaccinated, $P = 0.7$). Kaplan-Meier plots are shown in Fig. 1 for low expressors (Fig. 1E) and overexpressors (Fig. 1F). Importantly, there was a trend toward decreased mortality in vaccinated patients, most impressively seen in the low-expressor patients (control, 6.8%; vaccinated, 0.0%; $P = 0.08$). The mortality rate among patients with recurrent disease decreased in both vaccinated compared with control groups (overexpressors, 25% versus 50%; low expressors, 0% versus 38%). Overall follow-up times were similar in the low-expressor control and vaccinated groups but statistically longer in the overexpressor control group when compared with the vaccinated group ($P = 0.01$).

IHC Status Subset Analysis

Patients per IHC status. IHC status was known in 134 patients, including 57 in the control group and 77 in the vaccinated group. A comparable percentage of control and

Table 3. Demographics, prognostic factors, and treatment profiles of vaccinated HER2/*neu* overexpressor patients enrolled in E75 phase II trial by vaccine alone versus Tz + V

	Vaccine alone ($n = 22$)	Tz + V ($n = 7$)	P
Median age, range, y	52 (37-68)	54 (39-61)	0.9
Race, White/other, %	81.8/18.2	100.0/0.0	0.5
Tumor size, T_2-T_4, %	31.8	42.9	0.7
Histologic grade, grade 3, %	50.0	100.0	0.03*
NP, %	54.5	85.7	0.2
Hormone receptor negative, %	54.5	85.7	0.2
Chemotherapy, %	95.5	100.0	NS
XRT, %	72.7	85.7	0.6
Hormonal therapy, %	50.0	14.3	0.2

*Statistically significant difference.

vaccinated patients were in each IHC group (0, 8.8% versus 9.1%; 1^+, 26.3% versus 32.5%; 2^+, 42.1% versus 33.8%; 3^+, 22.8% versus 24.7%, respectively).

Demographics, prognostic factors, and treatment profiles per IHC status are detailed in Table 1B. As shown, IHC 1^+ patients had a larger percentage of T_2 to T_4 tumors in the control group compared with the vaccinated group (66.7% versus 30.8%; $P = 0.05$). IHC 3^+ control patients were all node positive, and 42.1% of vaccinated patients were node positive ($P = 0.003$).

Immunologic response per IHC status. Vaccination elicited an E75-specific *ex vivo* immune response in all IHC categories as shown by significant increases from prevaccination to maximum postvaccine E75-specific $CD8^+$ T cells (0, $P = 0.007$; 1^+, $P < 0.001$; 2^+, $P = 0.004$; 3^+, $P = 0.002$). Only IHC 1^+ patients showed a trend toward significant pre- to long-term increase in E75-specific $CD8^+$ T cells ($P = 0.08$; Fig. 2A).

In addition, patients were able to elicit an *in vivo* immune response as measured by DTH done pre- and postvaccination. Significant DTH increases were noted in all IHC categories (0, $P = 0.03$; 1^+, $P = 0.02$; 2^+, $P = 0.02$; 3^+, $P = 0.05$; Fig. 2B). Taken together, these data show that the E75 vaccine is immunologically effective, regardless of the extent of HER2/*neu* expression, but maybe most effective in IHC 1^+ patients.

Clinical response per IHC status. Disease recurrence and mortality are shown in Fig. 2C and D and Table 2B. In all IHC categories (except IHC 0, in which no patients recurred), downward trends in recurrence rates were observed when comparing control and vaccinated patients. More importantly, a significant decrease in mortality among vaccinated IHC 1^+ patients was identified: 20% mortality for control and 0% for vaccinated groups ($P = 0.05$). Overall follow-up times were similar in all groups except IHC 3^+, in which the trend for the control group was longer follow-up when compared with the vaccinated group ($P = 0.06$).

Trastuzumab before Vaccine (Tz + V)

Patients and safety. Most vaccinated overexpressor patients were enrolled before the establishment of trastuzumab as standard of care in the adjuvant setting. A total of 22 (76%) received the vaccine alone, and 7 (24%) received Tz + V sequentially. Both groups were comparable with respect to demographics and treatment profiles. They were also comparable with respect to prognostic factors except for nuclear grade. All patients receiving Tz + V had grade 3 tumors compared with 50% of those patients who received vaccine alone ($P = 0.03$; Table 3). Addressing the concern of additive local and systemic toxicity associated with combination HER2/*neu* directed therapies, we found no difference in toxicities between vaccine-alone and Tz + V patients (Fig. 3A).

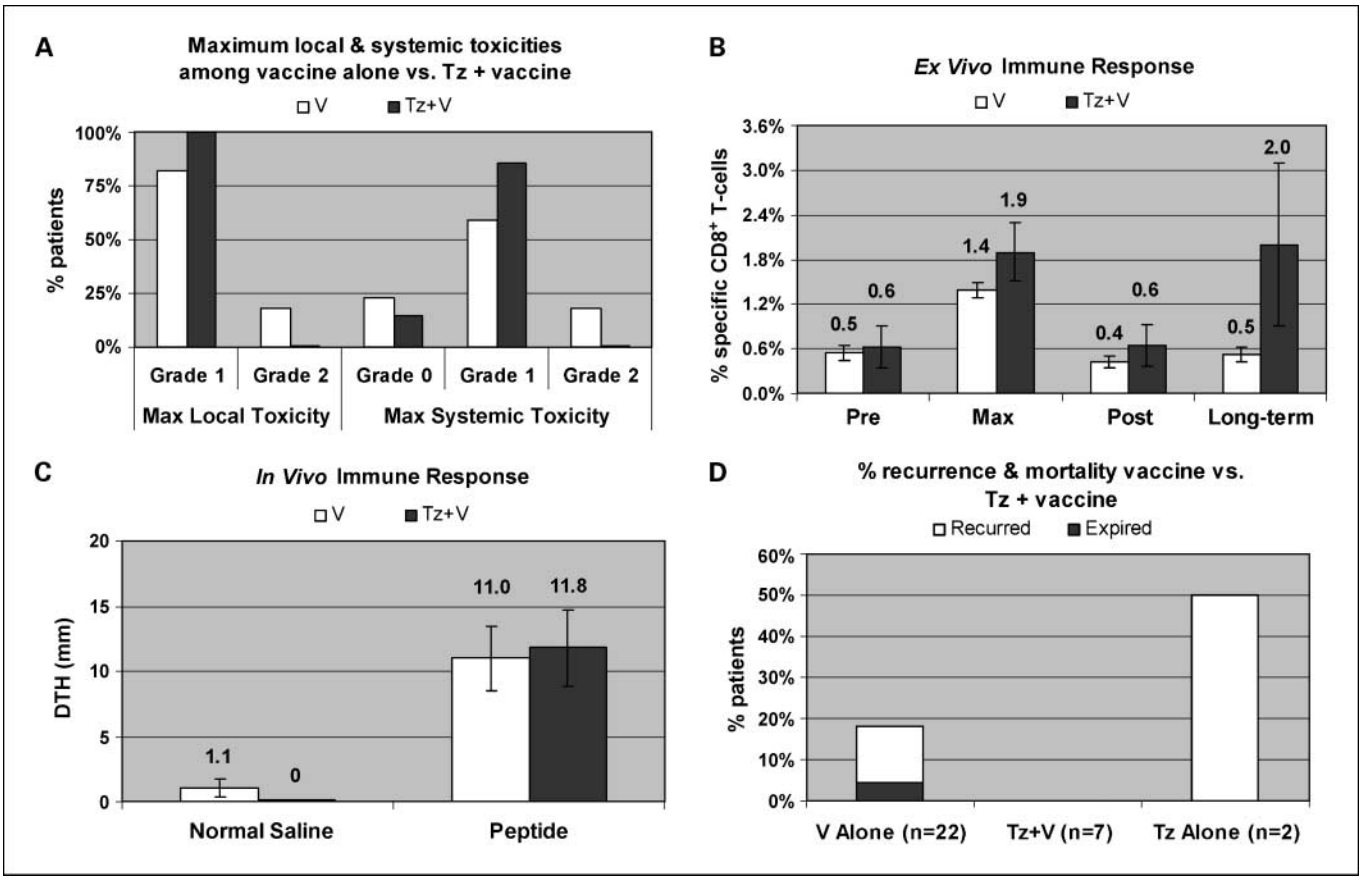

Fig. 3. Safety and immunologic response (*ex vivo* and *in vivo*) of HER2/*neu* overexpressor patients enrolled in E75 phase II trial who received vaccine alone compared with Tz + V. *A,* local and systemic toxicities: Tz + V did not increase either toxicity. *B, ex vivo* immune response (mean ± SE): both vaccine alone and Tz + V had statistically increased pre – maximum percentage specific $CD8^+$ T cells, and Tz + V had statistically significant increased long-term *ex vivo* response compared with vaccine alone ($P = 0.001$). *C, in vivo* immune response (mean ± SE): vaccine alone and Tz + V patients had increased postvaccine DTHs ($P < 0.001$ and $P = 0.008$, respectively). No difference was noted in vaccine alone versus Tz + V postvaccine DTH ($P = 0.8$). *D,* absolute recurrence and mortality rates of vaccine alone versus Tz + V versus trastuzumab alone.

Immunologic response Tz + V versus vaccine alone. Both groups of patients showed statistical increases in E75-specific $CD8^+$ T cells from prevaccine to maximum postvaccine ($P < 0.001$ and $P = 0.03$, respectively). The statistically significant difference in immune response was not apparent in the long term for vaccine-alone patients ($P = 0.2$) but persisted for Tz + V patients ($P = 0.05$). When compared with vaccine-alone patients, patients receiving the combination of Tz + V had statistically significant long-term E75-specific $CD8^+$ T cells ($P = 0.001$; Fig. 3B). Vaccine-alone and Tz + V patients had statistically significant increases in their postvaccine DTH responses when compared with their normal saline control ($P < 0.001$ and $P = 0.008$, respectively). However, no difference was noted between postvaccine DTH of vaccine alone versus Tz + V ($P = 0.8$; Fig. 3C).

Clinical response Tz + V versus vaccine alone. There were four recurrences and one death among vaccine-alone patients ($n = 22$) and no recurrences or deaths in the Tz + V group ($n = 7$). In addition, when assessing the control patients who received trastuzumab treatment (trastuzumab alone), there were two patients, one of these patients recurred and is still alive (Fig. 3D). Small sample size and low event frequency precluded statistical comparisons between these treatment groups.

Discussion

HER2/*neu* is a source of immunogenic peptides and is expressed in >75% of breast cancer patients. This protein is overexpressed in 25% of breast cancer, and these patients are candidates for trastuzumab immunotherapy. In a phase II clinical trial investigating the use of E75 as a preventive vaccine in high-risk breast cancer patients, our group has previously shown the vaccine to be safe, effective in eliciting an immune response, and clinically efficacious with decreased recurrence rates after a median follow-up of 20 months; however, this clinical benefit was lost because immunity waned without booster inoculations (23, 31).

In this article, we have shown that patients with all levels of HER2/*neu* expression as determined by IHC and FISH responded immunologically to E75 vaccination. Importantly, patients with low HER2/*neu* expression, specifically those with IHC 1^+ tumors, seemed to derive the greatest immunologic and clinical benefit. Interestingly, we have also shown that antigen-naïve patients (IHC 0) responded immunologically to the vaccine as well. Lastly, the sequential use of trastuzumab and the E75 vaccine seemed safe and may enhance the immunogenicity of the vaccine long term.

Before the routine use of trastuzumab (Food and Drug Administration approved for metastatic patients in 1998 and for adjuvant treatment in 2006), HER2/*neu* testing was not routinely used. The first patient in our E75 vaccine trial was enrolled in October 2000, and therefore, some of our patients had neither IHC nor FISH; however, most (92.1%) had either or a combination of IHC and FISH test done on their tumor specimens. In addition, our early overexpressor patients did not receive trastuzumab before 2006. In assessing the vaccine response by HER2/*neu* status, we attempted to determine the comparability of the control and vaccine groups. Some of the clinical benefit in the overexpressor group may be related to the number of node-positive patients in the control versus vaccinated arms because this was significantly higher; however, the low-expressor groups were completely comparable. In the low-expressor groups, we found that the vaccinated patients had a 41.2% reduction in recurrences and 100% reduction in mortality. This benefit highlights the difference in mechanism between the E75 peptide vaccine and trastuzumab. The latter, trastuzumab, has been shown to be less effective in low-expressor patients and is not indicated for use in this group, whereas the vaccine only requires protein expression, not overexpression (14). In fact, the immunologic data would suggest that low-expressor patients respond better to the vaccine than overexpressor patients, suggesting an element of immunologic tolerance in the overexpressor patients.

Many of the patients in this study, regardless of IHC status, had some element of pre-existing immunity as evident by prevaccine E75-specific $CD8^+$ T-cells levels of >0.3%. Even the antigen-naïve patients (IHC 0) had on average $0.5\% \pm 0.1\%$ E75-specific $CD8^+$ T cells, with five of the seven antigen-naïve vaccinated patients expressing pre-existing immunity (>0.3%). There are several possible explanations for this pre-existing immunity in purported antigen naïve patients. One possibility is that the IHC assessment was inaccurate because this assay is somewhat subjective or could have failed to provide a complete evaluation of the tumor specimens. Alternatively, the explanation may be immunoediting, the process of elimination, equilibrium, and escape described by Dunn and colleagues (32). Elimination, also known as cancer immunosurveillance, is responsible for destroying transformed cells; equilibrium occurs when new population of tumors cells with increasing mutations are present; escape is when tumor growth continues unrestrained by the immune system. This process suggests that HER2-positive tumors cells might have been eliminated. Evidence of ongoing immunosurveillance is also suggested in our study on healthy volunteers that showed rapidly inducible E75-specific cytotoxic T lymphocytes in 20% of healthy volunteers (33). In our previous trials, we have assumed that the peptide vaccine was amplifying a pre-existing immunologic response; however, in the case of truly antigen-naïve patients, the vaccine may be required to induce a response *de novo*. Ultimately, this is a crucial concept for the further investigation of E75 as a truly preventive vaccine in patients at high risk for first-occurrence breast cancer.

Trastuzumab has been shown to decrease recurrence rates and increase disease-free survival in the adjuvant setting in HER2/*neu* overexpressors, but this only applies to 25% of breast cancer patients (13). In addition, trastuzumab does not cross the blood-brain barrier and may be associated with increased incidence of cerebral metastases (34). Although the mechanism of action of trastuzumab is not completely elucidated, several mechanisms have been postulated to include direct blocking of the tyrosine kinase activity, immune activation of antibody-dependent cellular toxicity (35, 36), and inhibition of HER2 shedding by inhibiting metalloproteinase activity (37). Our laboratory and others have done preclinical studies with regard to the mechanism of trastuzumab and found that enhanced cell membrane turnover of HER2/*neu* occurs, which may lead to increased processing and presentation of immunogenic peptides and result in tumor cells that are more susceptible to peptide vaccine–induced killing (38). Our preclinical data and results from others suggest the combination of trastuzumab and peptide vaccination may be more efficacious than either agent alone, regardless of level of HER2/*neu* expression (39).

One concern for combination HER2/*neu*-directed immunotherapy is safety because a portion of trastuzumab patients develop cardiac toxicity. The mechanism for trastuzumab cardiac toxicity is unclear, but HER2/*neu* seems to affect myocyte survival (40). The concern about trastuzumab-induced cardiac toxicity has historically been greatest in patients receiving concurrent anthracyclines, but Buzdar and colleagues (41) have recently shown concurrent anthracyclines and trastuzumab to be safe in phase III trials. We show safety data in our seven patients with no increased local or systemic toxicity in sequential trastuzumab and E75 vaccination when compared with vaccine alone. In addition, Webster and colleagues (42) presented preliminary results of a phase I/II trial combining trastuzumab and a HER2/*neu* peptide vaccine in 14 stage IV breast cancer patients showing no increased cardiac toxicity. Both sets of data suggest improved immunogenicity in patients receiving combined treatment of trastuzumab and HER2/*neu* peptide vaccine.

In conclusion, our analyses would suggest that the HER2/*neu* low expressors, which represent >50% of breast cancer patients, may respond to a HER2/*neu* vaccine even if trastuzumab is not indicated for this group. We are pursuing two randomized phase III trials evaluating E75 peptide vaccine, one in low-expressor patients and the other in overexpressor patients following trastuzumab standard of care treatment, to further delineate the findings in this paper. In addition, in the current trial, we have vaccinated antigen-naïve patients and have found that this group of HER2/*neu* nonexpressors immunologically responds to the vaccine. We believe further trials are warranted in this group for the purpose of developing a truly preventive breast cancer vaccine. Lastly, trastuzumab and the E75 peptide vaccine seem to be safe for sequential use and may prove to be synergistic *in vivo* for overexpressors and potentially for low-expressor patients. We are conducting a phase I trial to further assess safety and immunogenicity in patients receiving concurrent combination HER2/*neu*-directed therapy with trastuzumab and a HER2/*neu* peptide vaccine.

Disclosure of Potential Conflicts of Interest

G. Peoples has inventorship rights in the E75 patent licensed to Apthera, Inc.

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Correction: Article on Impact of HER2/neu Expression Level on E75 Vaccine Response

In the article on impact of HER2/*neu* expression level on E75 vaccine response in the April 15, 2009 issue of *Clinical Cancer Research*, the name of an author, Ritesh Patil, was spelled incorrectly.

Benavides LC, Gates JD, Carmichael MG, et al. The impact of HER2/*neu* expression level on response to the E75 vaccine: From U.S. Military Cancer Institute Clinical Trials Group Study I-01 and I-02. Clin Cancer Res 2009;15:2895–904.

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The Impact of HER2/*neu* Expression Level on Response to the E75 Vaccine: From U.S. Military Cancer Institute Clinical Trials Group Study I-01 and I-02

Linda C. Benavides, Jeremy D. Gates, Mark G. Carmichael, et al.

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