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Advances in Brief

HER2/neu-derived Peptides Are Shared Antigens among Human Non-Small Cell Lung Cancer and Ovarian Cancer[1]

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Abstract

Previously, we have reported a correlation between the expression of *HER2/neu* and sensitivity to HLA-A2-restricted cytotoxic T-cells (CTL) in ovarian cancer. To investigate the role of *HER2/neu* in human non-small cell lung cancer (NSCLC), we established autologous tumor-specific CTL from tumor-infiltrating lymphocytes of HLA-A2$^+$ HER2/neu$^+$ NSCLC patients. These CTL lines specifically recognized HLA-A2$^+$ HER2/neu$^+$ autologous and allogeneic NSCLC cell lines as well as HLA-A2$^+$ HER2/neu$^+$ heterologous ovarian cancer cell lines. Furthermore, these CTL recognized an overexpressed, *HER2/neu*-derived peptide. From these results, we conclude that HLA-A2 serves as a restriction element in NSCLC. More importantly, at least one *HER2/neu*-derived peptide is a tumor-associated antigen in NSCLC and ovarian cancer.

Introduction

Tumor-specific CTL[3] have been detected in several human cancers (1, 2) and they could provide a therapeutic arm against malignant diseases in the form of a response to a tumor vaccine or through sensitization for subsequent adoptive immunotherapy. However, identification and characterization of TAA of human clinical tumors is required for optimal manipulation of CTL for clinical use. It would be even more beneficial for this purpose if we were to identify TAA which are shared by different tumors and not expressed by normal cells. In this sense, peptides derived from oncogene products may be potentially good targets for CTL. Recently, peptides derived from oncogene products such as p53 (3) and Ras (4, 5) have been shown to induce T-cell responses. We have previously demonstrated the relationship between *HER2/neu* expression and sensitivity to CTL in ovarian cancer (6). Peptide analysis revealed that a *HER2/neu*-derived peptide is one of the crucial T-cell epitopes recognized by HLA-A2-restricted, tumor-specific CTL, and this peptide is recognized in both ovarian and breast cancer.[4, 5] These findings have encouraged us to establish new therapeutic approaches to these malignancies. Lung cancer is one of the leading causes of death because of its frequency and its resistance to various therapeutic modalities (7), and therefore, poses a challenge for the development of novel therapeutic approaches to improve the prognosis of patients with this disease. Immunotherapy utilizing lymphokine-activated killer cells or TIL have been shown to be ineffective thus far (8, 9). In this study, we established CTL against NSCLC cells, and based on our previous results from ovarian and breast cancer, we examined if *HER2/neu* serves as a source of TAA in NSCLC since *HER2/neu* is also frequently expressed in NSCLC (10, 11).

Materials and Methods

CM. CM consisted of RPMI 1640 (BioWhittaker, Inc., Walkersville, MD) supplemented with 10% heat inactivated fetal calf serum (BioWhittaker), 2 mM L-glutamine (BioWhittaker), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (BioWhittaker), 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfate (BioWhittaker).

Preparation of TIL and Autologous Tumor Cells. TIL and autologous tumor cells were prepared as described previously (12). Briefly, fresh tumor tissues were excised from surgical specimens and minced into small pieces with scissors. This minced tissue was placed in a flask with a mixture of 0.1 mg/ml DNase type I, 1 mg/ml collagenase type IV, and 0.5 mg/ml hyaluronidase type V (all from Sigma, St. Louis, MO) in RPMI 1640 and stirred at room temperature for 2 to 4 h. The resultant cell suspension was washed in Hanks' balanced salt solution and subjected to Ficoll-Hypaque gradient (LSM, Organon Teknika, Durham, NC) centrifugation (1000 × *g*, 30 min). The interface was collected, suspended in CM, and subsequently subjected to two-layered (75 and 100%) Ficoll-Hypaque discontinuous density gradient centrifugation at 1000 × *g* for 20 min. Cells from 100% interface and 75% interface were used as TIL and tumor cells, respectively. TIL and tumor cells were also obtained from malignant pleural effusion or ascites with the use of the above discontinuous gradient centrifugation method.

Tumor Cell Lines. Non-small cell lung cancer cell lines (NCI-H460, NCI-H-647, NCI-H820, NCI-H1155, and NCI-H1355) were established and characterized in the NCI-Navy Medical Oncology Branch (Division of Cancer Treatment, National Cancer Institute, Bethesda, MD) (11). NCI-H460/NCI-H1155, NCI-H647, and NCI-H820/NCI-H1355 originate in large cell carcinoma, adenosquamous cell carcinoma, and adenocarcinoma, respectively. These lung cancer cell lines were cultured in the specially conditioned medium, ACL-4 (13). Ovarian epithelial tumor lines (Ov1, Ov2, and Ov3) were established in the Division of Surgical Oncology, Brigham and Women's Hospital (Boston, MA) from tumor specimens as described above. The expression of *HER2/neu* by these tumor cell lines was determined by Northern blotting analysis (11) and/or flow cytometry analysis, and the *HLA-A2* expression of the tumor cell lines was determined by flow cytometry analysis by using BB7.2 (anti-HLA-A2 mAb). *HER2/neu* expression determined by flow cytometry was matched with the results of Northern blot. Characteristics of each tumor cell line are summarized in Fig. 1. T2 is an HLA-A2$^+$ human mutant cell line which is defective in endogenous antigen presentation secondary to the deletion of the gene encoding TAP-1 and TAP-2 (14), and was used as a peptide-pulsed target in a chromium release assay.

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[3] The abbreviations used are: CTL, cytotoxic T-cells; CM, culture medium; NSCLC, non-small cell lung cancer; mAb, monoclonal antibody; TAA, tumor-associated antigen; TIL, tumor-infiltrating lymphocytes; FITC, fluorescein isothiocyanate.

[4] G. E. Peoples, P. S. Goedegebuure, R. Smith, D. C. Linehan, D. T. Hess, I. C. Summerhayes, I. Yoshino, and T. J. Eberlein. Breast and ovarian cancer specific cytotoxic T lymphocytes recognize the same *HER2/neu*-derived peptide, submitted for publication.

[5] D. C. Linehan, G. E. Peoples, D. T. Hess, I. C. Summerhayes, A. S. Parikh, P. S. Goedegebuure, and T. J. Eberlein. *In-vitro* stimulation of ovarian tumor associated lymphocytes with a mutated peptide derived from *HER2/neu* induces cytotoxicity against autologous tumor, submitted for publication.

CTL. HLA-A2$^+$ TIL obtained as described above were stimulated with solid phase anti-CD3 mAb (Ortho Pharmaceutical Corp., Raritan, NJ) for 48 h and expanded in CM containing 50 IU/ml recombinant interleukin 2 (Amgen, Thousand Oaks, CA) for 6 to 7 weeks. For the last 3 or 4 weeks, TIL were stimulated with irradiated autologous or allogeneic tumor cells weekly. We established CTL1/CTL2, CTL3/CTL4, and CTL5/CTL6 with stimulation of autologous non-small cell lung cancer cells, HLA-A2$^+$ allogeneic non-small cell lung cancer cells, and HLA-A2$^+$ allogeneic ovarian cancer cells, respectively. CTL1/CTL3/CTL5, CTL2, and CTL4/CTL6 were derived from three patients with adenocarcinoma, adenocarcinoma, and squamous cell carcinoma, respectively.

mAb. Anti-CD4 mAb (phycoerythrin-conjugated) and anti-CD8 mAb (FITC-conjugated) were purchased from Becton Dickinson (Mountain View, CA). Anti-HER2/neu mAb (TA-1) recognizing the extracellular domain of *HER2/neu* was purchased from Oncogene Science, Uniondale, NY. Culture supernatants of ATCC HB82 and ATCC HB96 (American Type Cell Culture Collection, Rockville, MD) were used as a source for anti-HLA DR mAb (L227) and anti-HLA-A2 (BB7.2), respectively.

Flow Cytometry. For direct single or two-color analyses, cells (5×10^5) were incubated for 30 min at 4°C with phycoerythrin- and/or FITC-conjugated mAb or murine control antibodies. In indirect staining, cells were incubated with the first mAb, or murine IgG (control) in staining medium (Hanks' balanced salt solution with 5% fetal calf serum) for 30 min at 4°C. After washing, cells were stained with FITC-conjugated anti-mouse IgG. The stained cells were fixed with 1% paraformaldehyde and kept at 4°C until analysis. Fluorescence analysis was performed by using a flow cytometer (Coulter, Hialeah, FL).

Cytotoxicity Assay. Cytotoxicity of CTL against tumor cells was examined by a standard ^{51}Cr-release cytotoxicity assay as described previously (12). Briefly, tumor targets were labeled for 1 h at 37°C with ^{51}Cr and washed. The target cells (2.5×10^3) were incubated with 2.5×10^4 to 1×10^5 of effector cells (effector:target ratio = 10:1 to 40:1) in 200 μl of CM in a 96-well round bottomed microtiter plate for 4 h at 37°C. The supernatant (100 μl) was collected and the samples were counted in a gamma counter (Gamma, Tnc 1191; TM Analytic, Elk Grove, IL). Percentage of specific lysis was evaluated by measuring the experimental minus the spontaneous divided by the maximum minus the spontaneous ^{51}Cr release.

Blocking of CTL Activity by mAb. Target cells were incubated with 1:10-diluted supernatant of L227-producing hybridoma or BB7.2-producing hybridoma for 30 min at 4°C after labeling with chromium. The mAb were added to the effector cells and target cells in 1:4-dilution, and a 4-h ^{51}Cr-release assay was performed as described above.

Synthesis of HER2/neu-derived Peptides. We synthesized the *HER2/neu*-derived peptide (654–662) with the sequence IISAVVGIL (GP2) and peptide (650–658) with the sequence PLTSIISAV (GP1) at the Biopolymer Laboratory at Brigham and Women's Hospital with an Applied Biosystems 430 peptide synthesizer. Both peptides have a point mutation at position 655 (valine to isoleucine) and contain a binding motif for the HLA-A2 molecule (15). This mutation creates the binding motif in GP2; however, the motif of GP1 is derived from the wild type and is not affected by this mutation. Crude products were purified on a C_{18} 4.6-mm inside diameter reverse phase high performance liquid chromatography and eluted with linear trifluoroacetic acid-acetonitrile gradients. Peptide was dissolved in aliquots of 1 mg/ml and stored at 4°C.

Statistical Analysis. Differences between cytotoxicities of CTL were examined by paired *t* test, and were considered significant when the *P* value was less than 0.05.

Results and Discussion

We successfully established autologous NSCLC-specific CTL (CTL1 and CTL2) by repetitive stimulation of TIL with irradiated autologous tumor cells following initial stimulation with immobilized anti-CD3 mAb and expansion in low-dose recombinant interleukin 2 (Fig. 1). Subsequently, we examined the cross-reactivity of the CTL against allogeneic *HER2/neu*-expressing tumor cells.

Both CTL1 and CTL2 showed cytotoxicity against the allogeneic HER2/neu$^+$ HLA-A2$^+$ NSCLC cell line, NCI-H1355, as well as autologous tumor cells, but there was no substantial killing against either HER2/neu$^+$ HLA-A2$^-$ NSCLC lines (NCI-H820) or HER2/neu$^-$ HLA-A2$^-$ lung large cell carcinoma cell lines (NCI-H460 and NCI-H1155) (Fig. 1*A*). CTL1 also killed HER2/neu$^+$ HLA-A2$^-$ lung adenosquamous cell carcinoma cell line (NCI-H647) (Fig. 1*A*); however, this cytolytic activity was not blocked by anti-HLA-A2 mAb (data not shown), indicating that this cytotoxicity is non-HLA-A2 restricted, and potentially an allogeneic response. Interestingly, CTL1 and CTL2 also killed allogeneic HER2/neu$^+$ HLA-A2$^+$ ovarian cancer cells (Ov1 and Ov3), while HER2/neu$^-$ HLA-A2$^+$ Ov2 was not killed (Fig. 1*B*). The cytolytic activity of CTL1 and CTL2 against autologous tumor cells, NCI-H1355 and Ov1, was blocked by anti-HLA-A2 mAb (Fig. 2). These data indicate that NSCLC-specific CTL recognize TAA in the context of HLA-A2 similar to ovarian cancer-specific CTL as previously reported (16). Furthermore, NSCLC appear to share common TAA with ovarian cancer, and our findings implicate HER2/neu.

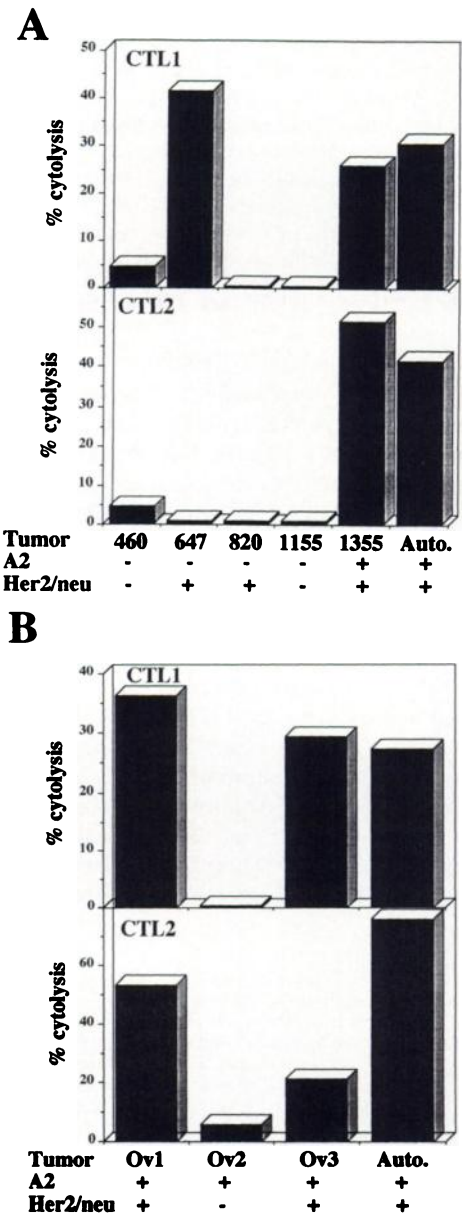

Fig. 1. Cross-killing of allogeneic tumor cells by NSCLC-specific CTL. (*A*) CTL1 and CTL2 were established from the TIL of two different patients by stimulation with autologous NSCLC (HER2/neu$^+$ HLA-A2$^+$), and tested for cytotoxicity against a panel of allogeneic lung cancer cell lines. (*B*) The cytotoxicity of CTL1 and CTL2 against allogeneic ovarian cancer cell lines was tested. The representative data from at least two experiments with similar results is shown.

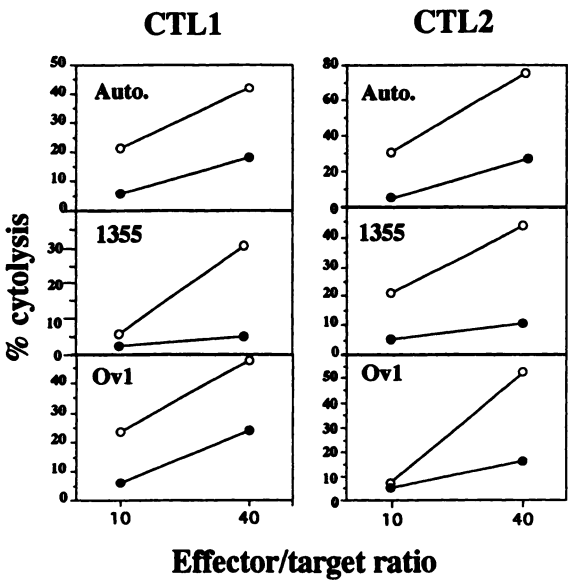

Fig. 2. Blocking effect of anti-HLA-A2 mAb on cytotoxic activity of CTL1 and CTL2. Cytotoxicity of CTL1 and CTL2 against autologous tumor cells, Ov1 and NCI-H1355 was examined in the presence of (●) or in the absence of anti-HLA-A2 mAb (○) in a chromium release assay. The representative data from at least two experiments with similar results is shown.

Next, we performed allogeneic tumor stimulation of NSCLC TIL and tested the cytotoxic profile of these CTLs. HER2/neu$^+$ HLA-A2$^+$ NSCLC (NCI-H1355)-stimulated CTL (CTL3 and CTL4) killed only HER2/neu$^+$ HLA-A2$^+$ targets (NCI-H1355 and Ov1), but not HER2/neu$^-$ (Ov2) or HLA-A2$^-$ (NCI-H820) target cells (Fig. 3). HER2/neu$^+$ HLA-A2$^+$ ovarian cancer stimulated CTL5 and CTL6 showed a similar pattern of cytotoxicity against the same targets (Fig. 3). The allogeneic stimulation was unsuccessful when the same TIL were stimulated by HER2/neu$^-$ or HLA-A2$^-$ tumor cells or when the TIL derived from HLA-A2$^-$ patients were stimulated by the HER2/neu$^+$ HLA-A2$^+$ tumor cells (data not shown). These data indicate that the cross-reactive signal is effective during the induction phase of the CTL.

To confirm that NSCLC TIL recognize a *HER2/neu*-derived antigen, we tested cytotoxicity of these CTLs against the T2 cell line pulsed with two *HER2/neu*-derived peptides (GP1 and GP2), both of which share a binding motif for HLA-A2. CTL1, CTL2, CTL5, and CTL6 killed GP2-pulsed T2 at a significantly higher level as compared with the killing of unpulsed T2 ($P < 0.05$), although the cytotoxicity of CTL3 and CTL4 against peptide-pulsed and unpulsed T2 was not significantly different (Fig. 4*A*). The cytolytic activities against T2 pulsed with GP2 were significantly blocked by anti-HLA-A2 ($P < 0.05$) (Fig. 4*B*). There was no substantial cytotoxicity against GP1-pulsed T2 among these CTL (Fig. 4*A*). From these results, we conclude that at least one *HER2/neu*-derived peptide (GP2) is a tumor antigen presented by HLA-A2 and recognized by NSCLC-specific CTL. Moreover, this tumor antigen is shared with ovarian carcinoma.

In this study, we used TIL obtained from clinical cases and succeeded in generating HER2/neu$^+$ tumor-specific CTL restricted by HLA-A2 by stimulation with irradiated tumor cells 3–4 times after activation with anti-CD3 mAb and expansion in low-dose recombinant interleukin 2. Disis *et al.* (17) reported that 10 stimulations were required to induce effective CTL directed against a HER2/neu-HLA-A2 complex when peripheral blood lymphocytes from normal donors and peptide-loaded antigen-presenting cells are used. These findings suggest that there are differences in the frequency of precursor CTL between TIL and lymphocytes from normal blood, and indicate the existence of circulating T-cells recognizing HER2/neu-derived peptides and their accumulation in tumor sites.

For therapeutic purposes, peptides recognized by CTL may be derived from *p53* (3), *Ras* (4, 5), and *HER2/neu* (17, 18), and thus have been investigated extensively in human and murine *in vitro* systems. *HER2/neu* is widely expressed and the overexpression of this gene accelerates tumor growth resulting in a poor prognosis in these patients (19, 20). We have demonstrated a HER2/neu-derived immunogenic peptide presented by HLA-A2 and recognized in ovarian cancer, breast cancer,[4, 5] and non-small cell lung cancer, suggesting that this antigen is widespread among human tumors. This antigen system may be theoretically useful in a peptide-based anti-tumor vaccine, as well as in the generation of anti-tumor CTL for use in

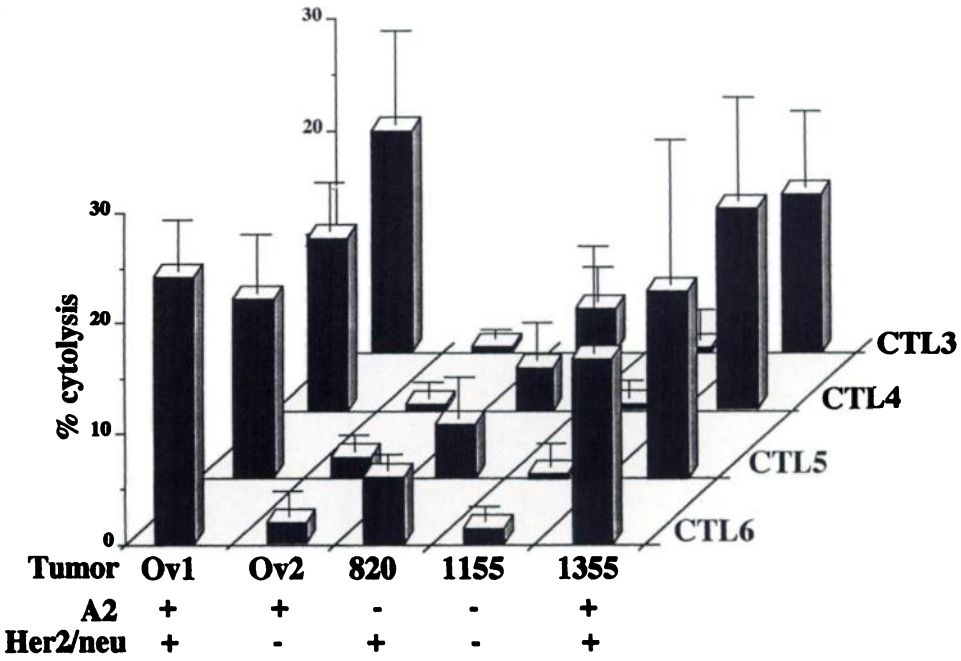

Fig. 3. Cytotoxic profile of CTL induced by allogeneic tumor stimulation. CTL3 and CTL4 were established by stimulation with NCI-H1355, and CTL5 and CTL6 were established by stimulation with Ov1. CTL3/CTL5 and CTL4/CTL6 were derived from the same patient, respectively. Cytotoxicity of these CTL against a panel of tumor cells was examined. The data shown is the mean ± SD calculated from 3 experiments.

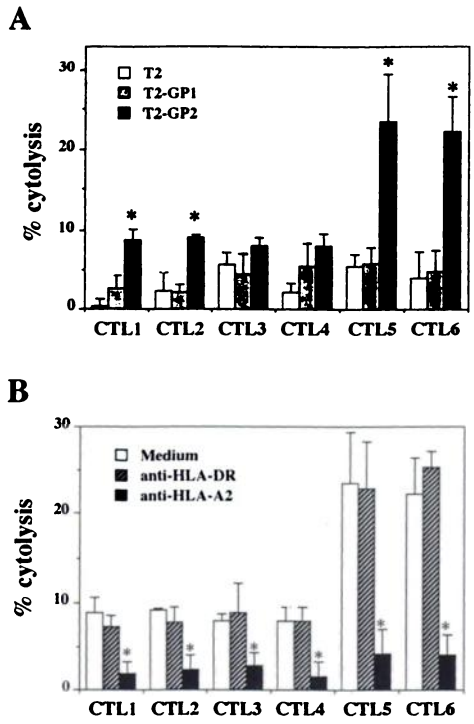

Fig. 4. Cytotoxicity of the CTL against T2 pulsed with HER2/neu-derived peptide. (*A*) Cytotoxic activity of a series of CTL against unpulsed T2, GP1-pulsed T2, and GP2-pulsed T2 was analyzed. *, significant differences ($P < 0.05$) as compared with killing of T2 by the same CTL. (*B*) HLA-A2 restriction was examined by looking at the blocking effect of anti-HLA-A2 mAb on the cytotoxicity of the CTL against GP2-pulsed T2. Anti-HLA-DR mAb was used as control. *, significant differences ($P < 0.05$) as compared with medium control.

adoptive immunotherapy.[5] In the present study, however, there was a discrepancy between the cytotoxicity against autologous tumor cells (Fig. 1) and that against GP2-pulsed T2 (Fig. 4) in both CTL1 and CTL2, and between that against HER2/neu$^+$ tumor cells (Fig. 3) and that against GP2-pulsed T2 (Fig. 4) in both CTL3 and CTL4, suggesting that *HER2/neu*-derived peptides presented to CTL in the context of HLA-A2 are diverse in NSCLC and ovarian cancer potentially related to differences in the level of expression of HLA-A2, *HER2/neu*, or other possible TAA among these tumors. Further investigation is required to clarify which peptides may be involved in different *HER2/neu*-expressing tumors.

Previously, immunotherapy has not been shown to be effective in lung cancer; however, the results presented in this study form the basis for further investigation into novel therapeutic modalities for NSCLC such as peptide vaccines and adoptive immunotherapy utilizing the *HER2/neu*-derived peptide-driven immune reaction.

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