Induction of HER2 Immunity in Outbred Domestic Cats by DNA Electrovaccination

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Abstract

Domestic cats share human living environments and genetic traits. They develop spontaneous feline mammary carcinoma (FMC) with similar histopathology to human breast cancer. HER2 and AKT phosphorylation was demonstrated in primary FMC by immunoblot analysis, indicating HER2 as a therapeutic target. FMC lines K12 and K248 expressing HER1, HER2, and HER3 were sensitive to receptor tyrosine kinase (RTK) inhibitors gefitinib and lapatinib. To test HER2 vaccine response in cats, purpose-bred, healthy cats were electrovaccinated with heterologous (xenogeneic) or point-mutated feline HER2 DNA. T-cell reactivity to feline self-HER2 was detected in 4 of 10 cats that received bare HER2, human–rat fusion HER2 (E2Neu) or mutant feline HER2 (feHER2-K), which contains a single amino acid substitution. The variable T-cell responses may resemble that in the genetically heterogeneous human population. All immune sera to heterologous HER2 recognized feline HER2 expressed in 3T3 cells (3T3/HER2), but not that in FMC K12 or K248. Immune sera to mutant pfeHER2-K bound 3T3/HER2 cells weakly, but they showed better recognition of K12 and K248 cells that also express HER1 and HER3, suggesting distinct HER2 epitopes displayed by FMC that may be simulated by feHER2-K. In summary, HER2 DNA electroporation overcomes T-cell immune tolerance in approximately 40% of healthy cats and induces antibodies with distinct specificity. Vaccination studies in domestic cats can expedite vaccine iteration to guide human vaccine design and better predict outcome, with the added benefit of helping feline mammary tumor patients.

Cancer Immunol Res; 3(7); 1–10. ©2015 AACR.

Introduction

Cancer vaccines hold great promise in disease treatment and prevention. However, the prolonged and costly process of regulatory approval, GMP production, and clinical trials prohibits efficient iteration between novel laboratory findings and human clinical trials. Test systems that can predict human immune responses will expedite vaccine development and cancer immunotherapy. Although murine models have been enormously successful in revealing basic immunology and providing proof of concept, they may fail short in predicting how the heterogeneous human population would respond to cancer vaccines (1–3). Transgenic mice expressing human tumor-associated antigens (TAA) and developing spontaneous tumors have produced more relevant results (2, 4, 5). We showed a striking difference in HER2 DNA vaccine response between BALB/c and C57BL/6 HER2 transgenic mice, illustrating the impact of genetic background on vaccine responses (6). Because a syngeneic mouse strain represents a single individual, each new vaccine should ideally be tested in multiple mouse strains representing diversified genetic backgrounds.

In transgenic mice, exogenous promoters are often used to drive the expression of TAAs, and antigenic peptides are usually presented by mouse rather than human major histocompatibility complex (MHC). T-cell repertoires are selected in the thymus in which both mouse and transgenic human antigens are presented, confounding the test system. The short life spans, lack of natural tumorigenesis, and comparatively small body weights are among the incongruities between rodents and humans. The limitations of these models bring clarity to the need for intermediate animal systems that more closely resemble those in humans, have similar cancer etiology, express a comparable repertoire of TAAs, and respond to the vaccine delivery approaches in a similar manner to humans. Importantly, immune responses in a genetically diverse outbred population would predict more accurately the range of immune responses in humans.

The domestic cat population is estimated at 1 billion worldwide (7) with approximately 95 million cats residing in U.S. households (8). Feline whole-genome sequencing and SNP maps have been generated (7, 9), revealing homologous genetic diseases between humans and cats (10). Pet animals share human dwellings and develop cancers similar to those that develop in humans. Domestic dogs and cats develop spontaneous mammary tumors. The incidence of mammary tumor is higher in dogs (11, 12), but half of canine mammary tumors are benign, whereas 90% of feline mammary tumors are malignant and mostly adenocarcinomas (13–15). Hormonal regulation of canine and feline mammary tumors is evident because the incidence is drastically reduced by ovariohysterectomy (12, 16). About 15%
of nonspayed domestic cats develop spontaneous mammary carcinoma with disease progression and histopathology similar to that of human breast cancer, and HER2 expression has been reported (14, 17–24). Feline mammary carcinoma (FMC) patients are treated with surgery, radiotherapy, and chemotherapy. Thus, domestic felines represent a particularly suitable model for evaluating breast cancer vaccines.

DNA vaccines have advanced more expeditiously in veterinary medicine than in human medicine. The first approved therapeutic cancer vaccine in any species was a plasmid DNA encoding heterologous (xenogeneic) human tyrosinase for treatment of canine oral melanoma (14, 25, 26). Two other DNA vaccines targeting West Nile virus in horses (27) and hematopoietic necrosis virus in salmon (28) have also been approved for veterinary use. In this study, intramuscular electroporation was used to evaluate novel feline HER2 DNA vaccines because the procedure is well tolerated by humans (29, 30), and gene expression is dramatically enhanced by electroporation (29, 31–34). HER2 DNA vaccines that we have developed have been tested in human clinical trials, in which a kinase-deficient full-length human HER2 DNA induced T-cell responses in breast cancer patients (35–37). We reported another HER2 construct expressing the wild-type (WT) human extracellular and transmembrane domains (36, 37), which was also tested in breast cancer patients, but without evidence of T-cell responses (38). A human HER2/rat neu hybrid vaccine showed improved efficacy in transgenic mice (4, 39), and a trial is currently open for accrual. In the present study, heterologous HER2 as well as feline HER2 with a single amino acid (a.a.) substitution were tested in outbred domestic shorthair cats with an aim to establish an improved system for predicting cancer vaccine response in humans.

Materials and Methods

Animals and tissues

BALB/c mice were purchased from Charles River Laboratory. Pathogen-free (SPF) purpose-bred domestic shorthair cats ages 6 months to 2 years were obtained from Liberty Research, Inc. Animals were housed and maintained in the Department of Laboratory Animal Resource facility at the Wayne State University School of Medicine in accordance with Institutional Animal Care and Use Committee guidelines. The experimental cats were adopted as domestic pets by the caretaker community after completion of the study. A black bear legally harvested in Ontario, Canada, was the donor of the liver tissue.

FMC samples were obtained from mastectomy tissues of 2 feline patients treated at Oakland Veterinary Referral Services (OVRS) in Michigan with consent from the cat owners (Supplementary Table S1). OVRS-1A and OVRS-1B are two independent primary tumors from the same cat. Three additional mammary tumor samples with paired, uninvolved stromal tissues were purchased from Colorado State University (CSU-133, 418, and 1646).

Cell lines

The K248 cell line was established from a pulmonary metastasis of a Siamese cat that had a mammary carcinoma, and was provided by Dr. William Hardy Jr, and provided by Dr. Jaime Modiano of the University of Pennsylvania (40). SKOV3 cells were purchased from the ATCC. MCF7 cells were obtained from Lisa Polin of the Karmanos Cancer Institute. All cells were maintained in DMEM supplemented with fetal bovine sera, penicillin, and streptomycin. The feline origin of K248 and K12 cells has been authenticated by short tandem repeat analysis of four loci (Supplementary Fig. S1).

Immunohistochemical analysis

Pathologic diagnoses were performed according to the WHO classification for tumors in domestic animals. For feline HER2 detection, epitopes were retrieved with sodium citrate buffer (pH 6.0) and histologic grade primary antibodies were applied according to the manufacturer’s recommendation (HER2, clone Z4881; Invitrogen) followed by broad-spectrum HRP polymer conjugate (SuperPicTure Polymer Detection Kit; Zymed) and DAB substrate (Pierce Biotech). Feline mammary tumor cells, K248, were injected s.c. into SCID mice. Tumor explants were used as controls.

DNA cloning and construction

All cloning primer sequences are shown in Supplementary Table S2. Feline ERBB2 DNA encoding HER2 was cloned from cell lines K248 (40) and K12 (41), and from the ovary of a domestic shorthair cat using a Protoscript kit (New England Biolabs), all of which showed identical sequences. The confirmed full-length feline ERBB2 (fHER2) cDNA sequence has been submitted to Genbank (JN990983). For vaccination, a stop codon was introduced after codon 687 to delete the oncogenic intracellular domain, then subcloned into pVax1; the resultant pFHER2 contains the signal peptide, and the extracellular and transmembrane domains of feline HER2. The pFHER2-K contains a single-nucleotide substitution in codon 141 (cag → aga) of the extracellular domain (ECD) I, based on our reported sequence of feline ERBB2 (Genbank JN990983) and was generated by PCR-based methods and verified by DNA sequencing.

Black bear ERBB2 DNA encoding black bear HER2 was similarly cloned from the liver tissue of a black bear. The cDNA sequence was submitted to Genbank (JQ045008). DNA vaccine pBHER2 encoding the signal peptide and the extracellular and transmembrane domains were constructed by PCR-based methods similar to that described for pFHER2.

Feline CSF2 cDNA encoding feline GM-CSF was amplified from a randomly primed cDNA library (Protoscript; New England Biolabs) prepared from ConA-stimulated feline peripheral blood mononuclear cells (PBMC) and cloned into pCDNA3.1. The open reading frame sequence was in accord with the consensus of feline CSF2 cDNAs in Genbank (AY878357, NM_001009846, AF053007, and AY138140).

Generation of recombinant feline HER2 and human Fc fusion protein—fHER2-Fc

A plasmid encoding the secreted fusion protein of feline HER2 ECD fused to human Ig Fc was constructed. Feline ERBB2 cDNA and the human Ig Fc domain (IGHG1, Genbank BC080557) were PCR amplified and fused by overlap-extension priming, giving a 2,680-bp product, which was cloned into the HindIII and Xbal sites of pVax1. Murine 3T3 cells were transfected with this construct, and the fHER2-Fc protein in culture supernatant
was quantified by ELISA using mouse anti-human HER2 capture mAb (clone TA-1; Calbiochem), which cross-reacts with feline HER2. Rabbit anti-human IgG was the detection antibody (Jackson ImmunoResearch). huHER2-Fc was purchased from Sino Biologics.

**Electrovaccination of mice and cats**

Mice were injected with an admix of 50 µg each of vaccine plasmid and plasmid encoding murine GM-CSF (pnuGM-CSF) in 50 µL PBS in the gastrocnemius muscle (42). Conductive gel was applied on the skin over the injection sites. Electroporation was conducted with NEPA21 electroporator (Napagene) using a tweezer electrode. Three 50-ms degenerating bipolar pulses of 100 V were administered at each site. Cats were injected with 1.5 mg each of HER2 vaccine plasmid and pFpGM-CSF in 1.5 mL PBS, divided equally over three injection sites in the biceps femoris or quadriceps. Two rounds of electroporation were applied to each site as described using a 1.3-cm² caliper electrode (BTX).

**Cell proliferation assay**

Cells were plated at 2,000 to 5,000 per well in 96-well plates and treated with gefitinib or lapatinib in quintuplicate for 48 hours. Alamir Blue reagent (Life Technologies) was added and fluorescence measured after 3 to 4 hours. The percentage of proliferative activity was determined relative to the average of untreated samples.

**Western blot analysis**

Cells or tissues were lysed in a nonionic detergent lysis buffer (43) with protease inhibitor cocktail (Roche Diagnostics) immediately after the addition of phosphatase inhibitors (NEB). Total protein was quantified by BCA assay (Pierce Biotech). Protein (10 µg) was boiled in Laemmli buffer, separated onto polyvinylidene difluoride membrane for overnight incubation with antibody to HER2 (42/c-erbB-2; BD Biosciences), phospho-HER2 Y1248 (polyclonal; Cell Signaling Technology), Akt (polyclonal; Cell Signaling Technology), phospho-Akt S473 (587F11; Cell Signaling Technology) or β-actin (I-19; Santa Cruz Biotechnology). After washing in TBS-Tween, membranes were incubated with horseradish peroxidase (HRP)–conjugated secondary antibody before washing and development using enhanced chemiluminescent reagents (Thermo Scientific).

**Flow cytometric analysis**

HER2/neu epitopes were detected by mAb TA-1 (Calbiochem), trastuzumab (Genentech), 7.16.4 (Calbiochem), N12, or N29 (hybridoma cell lines were generous gifts of Dr. Yosef Yarden, Weisman Institute, Israel). mAb to human EGFR (528; Santa Cruz Biotechnology), HER3 (SGP1; ebioscience) and HLA-ABC (W6/32; ebioscience) were used as indicated. Phycoerythrin (PE)–conjugated goat anti-mouse or anti-human IgG was the secondary antibody (Jackson ImmunoResearch). Flow cytometric analysis was performed using FACS Canto II and data analyzed with FlowJo (Tree Star).

To measure the antibody level in immune sera, mouse or feline sera were incubated with 3T3 cells engineered to express the designated antigen and detected by PE-conjugated anti-mouse or feline IgG secondary antibody (Santa Cruz Biotechnology). Mouse antibody concentrations were extrapolated from a standard curve of HER2 mAb TA-1. Feline antibody titers were determined by serial dilution until binding was no longer detected above that of the isotype control.

**Analysis of T-cell response by ELISPOT**

Mouse splenocytes or feline PBMCs isolated by ficoll separation (GE Healthcare) were maintained in RPMI medium supplemented with fetal bovine sera, penicillin–streptomycin. Feline PBMCs were supplemented with 0.5 µg/mL feline IL2 (R&D Systems). Cells were plated at 2 × 10⁵ per well in round bottom 96-well plates and cultured with 10 µg/mL fHER2-Fc (3T3 supernatant-equivalent as described above), huHER2-Fc, human IgG control (Jackson ImmunoResearch) or control 3T3-conditioned medium for 48 (mouse) or 72 (feline) hours. Total well contents were then transferred to mouse or feline (R&D Systems) IFNγ ELISPOT plates and incubated for an additional 48 hours before detection and enumeration as previously described (44) or per the manufacturer’s protocol. Visualized cytokine spots were enumerated using the ImmunoSpot analyzer (CTL) and expressed as the number of cytokine-producing cells per 10⁶ splenocytes or PBMCs.

**Results**

**Expression of HER2 in FMC**

Expression of ERBB family receptor tyrosine kinase (RTK) in FMC was measured by flow cytometry. Surface staining of HER1 (EGFR), HER2, and HER3 was detected in K12 and K248 cells using mAbs to their human homologs (Fig. 1A). MHC I expression was also detected with mAb W6/32 to a constant region of HER2 DNA Vaccination of Domestic Cats

**Figure 1.** Expression of HER2 in FMC. A, flow cytometric analysis of cell surface EGFR (HER1), HER2, HER3, and MHC I expression (open histograms) in FMC cell lines K12 and K248. Human SKOV3 cells are included as controls. Shaded histograms are unstained controls. B, immunohistochemical analysis of 3 primary FMC samples and FMC line K248 outgrowth in SCID mice using polyclonal Ab to huHER2. H&E is shown in parallel. All images are at ×40 magnification.
human MHC I. Control human ovarian cancer cell line SKOV3 showed elevated HER1 and HER2, while lacking HER3 expression (45). Feline HER2 expression in primary FMC clinical samples (Supplementary Table S1) was detected by immunohistochemical (IHC) staining (Fig. 1B). Membrane staining of HER2 was detected in all 3 primary FMC samples and in K248 explant, consistent with membrane staining of K248 cells by flow cytometry (Fig. 1A). Membranous HER2 in FMC would permit recognition by both humoral and cellular immunity. Cytoplasmic staining was also detected in OVRS-1A and the K248 explant, and may indicate accumulation of incompletely or incorrectly processed HER2. The clinical significance of cytoplasmic HER2 remains unclear, but T cells would recognize peptides derived from surface or cytoplasmic HER2.

**RTK activity in FMC**

Activation of RTK signaling in FMC was tested using a human RTK array (R&D Systems; Supplementary Fig. S2). Although cross-reactivity with feline antigens by all antibodies in this array was not verified, AKT phosphorylation (S473) was elevated in 3 of 3 FMC tissue samples—CSU-133, 418, and 1646—compared with their paired, uninvolved stromal tissue. AKT phosphorylation was also observed in K12 and K248 cells, consistent with RTK pathway activation in FMC. HER2 (Y1248) and downstream AKT (S473) phosphorylation in primary FMC tissue was further tested by Western blotting (Fig. 2A). Total and phosphorylated HER2 and AKT were detected in FMC OVRS-2, CSU-133, CSU-418, and CSU-1646, demonstrating activation of HER2 and downstream RTK signaling events.

To further test whether ERBB RTK signaling is required for FMC cell proliferation, FMC cell lines K12 and K248 were cultured with or without ERBB family tyrosine kinase inhibitors gefitinib or lapatinib (Fig. 2B). Both K12 and K248 exhibited dose-dependent inhibition of cell proliferation. SKOV3 and MCF7 cells were the positive and negative control, respectively. Therefore, FMCs express functional tyrosine kinase receptors that trigger downstream signaling and cell proliferation.

**Cloning and characterization of feline HER2**

Feline ERBB2 cDNA, encoding HER2, cloned from K12, K248, and normal ovary showed identical sequences (GenBank Accession JN990983). The a.a. translation of the full-length feline HER2 shared 93% sequence identity with human HER2 (Fig. 3A and Supplementary Fig. S3). Black bear HER2 (GenBank Accession JQ040508) had 96% and 92% a.a. sequence identity with feline and human HER2, respectively. The feline, black bear, human, and rat HER2/neu extracellular and transmembrane regions (EC/EM) were individually transfected into 3T3 cells. Epitope expression was compared by staining with mAbs to human HER2 (TA-1, N12, N29, and trastuzumab) or rat neu (7.16.4; Fig. 3B; refs. 46–49). Feline HER2 was recognized by all 5 mAbs. The closely related black bear HER2 was recognized by 4 mAbs, except trastuzumab, signifying structural disparity at this epitope. mAb 7.16.4 identifies a rat neu epitope, which is present in feline and black bear, but not in human HER2. Overall, there are high levels of epitope sharing among HER2 molecules from these four species, with cat HER2 expressing all 5 epitopes recognized by the panel of mAbs.

We previously showed that heterologous rat neu electrovaccination overcame T-cell tolerance in human HER2 transgenic (Tg) mice, but immune sera to neu did not recognize human HER2 (41). A human HER2-rat neu hybrid vaccine (pE2Neu) encoding human HER2 ECD domains I/II, rat neu domains III/IV, and neu transmembrane domain induced both humoral and cellular immunity in HER2 Tg mice (4). On the basis of these findings, heterologous bear, human and rat HER2/neu genes sharing high levels of sequence identity with feline HER2 were tested as candidate vaccines. To evaluate whether minimal alteration could be engineered to afford immunogenicity while preserving feline HER2 epitopes, a single a.a., glutamine-141 in domain I of the feline HER2 ECD, was replaced with lysine to generate fHER2-K. Supplementary Fig. S4A and S4B show the predicted three-dimensional structure of fHER2-K domains I–III using cartoon and space-filling models. The predicted effect of substituting Q with K at a.a. 141 is portrayed by an electrostatic surface model (SYBYL-X...
A

Full-length HER2 BLAST analysis (%identities/%positives/%gaps)

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B

Figure 3.

HER2 sequences and epitopes. A, BLAST analysis of full-length HER2 a.a. sequence identity. B, schematic of binding by 4 mAbs to human HER2 (TA-1, N29, N12, and trastuzumab), and 1 mAb to rat neu (7.16.4). 3T3 cells transfected to express the indicated HER2 ECTM were stained with 1 μg/mL of the indicated mAb (open histogram) with detection by PE-conjugated secondary antibody. Secondary antibody alone (shaded histogram) was the negative control.

2.1.1 software; Tripos), in which the electropositive side chain of K is indicated by red and Q is shown in blue (Supplementary Fig. S4C).

Immunogenicity of feHER2-K

Recombinant feHER2-K was expressed in 3T3 cells and characterized by flow cytometry (Fig. 4A). Positive staining by the panel of 5 mAbs indicated preservation of 5 HER2/neu epitopes after the Q→K substitution. Improved binding of feHER2-K by neu-specific mAb 7.16.4 compared with its binding to WT feHER2 (Fig. 3B) suggests a possible structural alteration of this epitope.

Immunogenicity of feHER2-K and feHER2-K was initially tested in BALB/c mice by electrovaccination with an admix of pfeHER2, pfeHER2-K, or control phuHER2 (encoding human HER2 ECTM) together with pmuGM-CSF encoding murine GM-CSF (4, 6, 42, 50). After two immunizations, mice produced 59 ± 19, 49 ± 13, and 39 ± 20 μg/mL IgG to their cognate antigens, respectively, as measured with 3T3 cells transfected with individual test antigens (Fig. 4B). Expression of feHER2, feHER2-K or huHER2 on individually transfected 3T3 cells was comparable as verified with mAb TA-1 binding at two different concentrations (Supplementary Fig. S5A). pfeHER2 or pfeHER2-K antibodies are highly cross-reactive, indicating the recognition of dominant foreign epitopes on feline HER2 (Fig. 4B, left and middle). The Q141K substitution appears to create only subtle changes that did not alter the immune response to the dominant foreign epitopes. Modest cross-reactivity between feline and human HER2 immune sera was also observed. Neither pre- nor post-vaccination mouse serum bound untransfected 3T3 cells (Supplementary Fig. S5B), supporting the HER2 specificity of the immune sera.

To measure T-cell response, a feline HER2 ECD and human Ig Fc fusion protein, feHER2-Fc, was generated as the test antigen and verified by Western blot analysis (Fig. 4C). Recombinant human HER2-Fc (huHER2-Fc) and SKOV3 cell lysate were the controls. Splenocytes from immunized BALB/c mice were incubated with feHER2-Fc or control huHER2-Fc and IFNγ-producing T cells were enumerated by ELISPOT. T-cell responses to feline HER2 were induced by vaccination with pfeHER2, pfeHER2-K, or phuHER2, showing cross-reactivity between feline and human HER2 antigen (Fig. 4D). This finding also indicates that the Q141K substitution does not alter mouse T-cell response to the WT HER2 protein. More prominent responses were observed after in vitro stimulation with huHER2-Fc. This result may reflect a more effective stimulation with purified huHER2-Fc protein versus feHER2-Fc in culture supernatant.

DNA electrovaccination of cats

For feline vaccinations, GM-CSF cDNA was cloned from feline PBMCs (pfgm-CSF). Generation of recombinant GM-CSF in the supernatant of transfected 3T3 cells was verified by Western blot analysis (R&D Systems, Inc.; data not shown). The feasibility of DNA electrovaccination in cats was initially tested with pE2Neu encoding a fusion protein of HER2 and neu (4). Three healthy purpose-bred, pathogen-free domestic shorthair cats 12 to 24 months of age were injected with pE2Neu and pfgm-CSF in three legs in the biceps femoris or quadriiceps following by electropropagation. Vaccination was administered four times at 3-week intervals. Blood was collected through the jugular vein 2 weeks after each vaccination. Human HER2 and rat neu binding antibodies were measured by flow cytometry. Human HER2 binding IgG reached a titer of 1:400,000 in 2 cats and 1:100,000 in the other (Fig. 5A, left). Rat neu binding Ab ranged from 1:25,000 to 1:100,000 (Fig. 5A, middle). Vaccination with pE2Neu induced antibody that also recognized 3T3 cells expressing WT feline HER2 (Fig. 5A, right), showing reactivity to self-HER2. Neither pre- nor post-fourth vaccination feline serum stained untransfected 3T3 cells, illustrating the HER2/neu-specific reactivity of the immune sera (Supplementary Fig. S6A).

Immune response to novel HER2 DNA vaccines

A panel of HER2 vaccines was tested in 15 additional healthy cats between 5 and 8 months of age. Cats were electrovaccinated four times with pfeHER2, pfeHER2-K, pheAR2, pE2Neu, or an admix vaccine of pfeHER2-K and pE2Neu. As observed previously, the 6 cats that received pE2Neu either alone or in combination with pfeHER2-K developed high levels of IgG antibody to human HER2 peaking after three vaccinations (Fig. 5B, left). pheAR2 immune sera cross-reacted with human HER2 and the titers increased after each booster immunization. pfeHER2 or pfeHER2-K immunization did not induce significant antibody response to human HER2.

Recognition of feHER2-K by immune sera was measured by their binding to 3T3/feHER2-K (Fig. 5B, middle). A prominent response (~1:120,000) was induced by pfeHER2-K+pE2Neu.
Individually, pFHER2-K, pE2Neu, or pBearH2 induced moderate titers averaging approximately 1:6,000, a 10- to 20-fold reduction from the admix vaccine, suggesting a synergistic or adjuvant effect of the heterologous pE2Neu. pFHER2 vaccination did not generate a significant antibody response to pFHER2-K.

Binding to 3T3 cells expressing WT feline HER2 (3T3/feHER2) was detected between 1:1,600 and 1:3,200 with immune sera from pE2Neu, pFHER2-K-pE2Neu, or pBearH2 vaccinated cats (Fig. 5B, right) and may be a result of antigenic cross-reactivity between heterologous HER2 and feline HER2. pFHER2 and pFHER2-K immune sera showed negligible binding to 3T3/feHER2. The specificity of HER2 antigen recognition was validated by the absence of binding to untransfected 3T3 cells after four vaccinations (Supplementary Fig. S6B).

The reactivity of immune sera was further tested at 1:50 to 1:100 dilution using 3T3/feHER2 that express only feline HER2 as well as K12 and K248 cells that express HER1, HER2, and HER3, allowing HER2 to be heterodimerized (Fig. 5C). pFHER2 immune sera showed little or low-level binding to 3T3/feHER2, but significant binding to K12 and K248 cells (Fig. 5C, middle and right). Although other immune sera recognized 3T3/feHER2, they reacted minimally with K12 or K248 cells. These findings may suggest that pFHER2-K displays epitopes that mimic those exposed naturally on FMC cells, and warrant further investigation.

### T-cell response to HER2 DNA vaccines

T-cell response to foreign huHER2 was measured first. Immune PBMCs were cultured with recombinant huHER2-Fc for 3 days before IFNγ ELISPOT analysis (Fig. 6A). The 6 cats receiving pE2Neu with or without pFHER2-K responded to human HER2 at 1:30 to 750 SFU/10⁶ PBMCs, validating the effectiveness of DNA electrovaccination (Fig. 6A). pBearHER2 immune T cells also cross-reacted with human HER2, producing an average of approximately 160 SFU/10⁶ PBMCs. Vaccination with pFHER2-K alone did not induce T cells that recognized human HER2.

IFNγ T-cell response to pFHER2-Fc was measured to evaluate reactivity to self-HER2 (Fig. 6B). Of the 10 cats evaluated, 3 produced significant pFHER2-specific T-cell responses, with 1 cat each from the pFHER2-K (~100 SFU/million cells), pE2Neu (~270 IFNγ spots), and pBearHER2 (~280 IFNγ spots) groups. The observed T-cell responses indicate the immunogenic nature of both heterologous and point-mutated HER2 vaccine. The 30% response rate may reflect the heterogeneous genetic background of outbred cats, as in humans.

Three bi-weekly booster vaccinations were given to 5 cats that received pFHER2-K or pFHER2-K-pE2Neu (Fig. 6C). Only 1 of the 3 cats receiving the admixed vaccine converted from a non-responder to a responder after three boosters (~135 SFU/10⁶ PBMCs). Therefore, a 40% T-cell response rate to self-HER2 was achieved in healthy cats. Those cats initially receiving pE2Neu or pBearHER2 were boosted three times with pFHER2-K (Fig. 6D and E). Responses to recombinant human or feline HER2 did not increase, suggesting that cross-reactive T cells, not common epitopes in HER2 from pE2Neu or bear HER2, contributed to feline HER2 reactivity.

The cats tolerated the vaccination procedure without signs of pain or discomfort after recovery from anesthesia. No adverse side effects were detected 6 to 12 months after the final vaccination and the cats continue to thrive.

### Discussion

To the best of our knowledge, this is the first report of cats receiving DNA electrovaccination and the induction of HER2 immunity by self-HER2 with a single a.a. substitution. The expression of HER1, HER2, and HER3 and the sensitivity to RTK inhibitors in FMC lines, combined with HER2 and AKT...
phosphorylation in primary tumor samples, demonstrate RTK activity in FMC. Together, with their hormone responsiveness and histopathology, feline mammary tumors naturally resemble human breast cancer. All cats responded to foreign antigen via DNA electroporation to validate the feasibility of this immunization modality. In 2 of 5 healthy cats, vaccination with pE2Neu or p\(\text{bearHER2}\) overcame immune tolerance to trigger T-cell responses to self-\(\text{feHER2}\). Vaccination with p\(\text{feHER2-K}\) also induced \(\text{feHER2}\)-specific T-cell responses in 1 of 3 cats. Additional studies will be necessary to validate and enhance T-cell activation by feline \(\text{HER2}\) vaccines. Antibody binding to 3T3/\(\text{feHER2}\) was detected in cats vaccinated with heterologous HER2. Although p\(\text{feHER2-K}\) immune sera were the least effective in binding 3T3/\(\text{feHER2}\), these immune sera recognized unique epitopes on FMC K12 and K248. Taken together, outbred cats represent a new model system for evaluating novel \(\text{HER2}\) DNA vaccines. Importantly, the vaccine design and feline test system can be extrapolated to other tumor-associated self-antigens to expedite the development of veterinary and human cancer vaccines.

\(\text{feHER2-K}\) immune sera appear to bind epitopes on 3T3/\(\text{feHER2-K}\) as well as on K12 and K248 FMC, but only minimally to 3T3/\(\text{feHER2}\). Although other interpretations may exist, we propose the following scenario: \(\text{feHER2-K}\) recapitulates a cryptic \(\text{HER2}\) epitope naturally displayed when WT \(\text{feHER2}\) is heterodimerized or associated with other ERBB family members. Cats immunized with heterologous pE2Neu or \(\text{p\(\text{bearHER2}\)}\) produced antibodies that recognize dominant epitopes on WT \(\text{feHER2}\) as well as \(\text{feHER2-K}\) expressed on 3T3 cells, but not the cryptic epitope exhibited by K12 or K248 cells. This seemingly subtle, but potentially critical difference in \(\text{HER2}\) epitope recognition was made possible by vaccinating genetically unmanipulated cats that naturally express \(\text{HER1}, \text{HER2}, \text{and HER3}\). It will be important to further evaluate immune sera from feline \(\text{HER2}\) vaccines, engineered with modifications to specific domains, may help bring clarity.

Our previous study showed consistent responses to \(\text{HER2}\) DNA electrovaccination in BALB/c \(\text{HER2 Tg}\) mice, but poor responses in...
C57BL/6 HER2 Tg mice (4, 42), illustrating the impact of genetic background on vaccine response. The overall 30% to 40% T-cell response rate in cats is deemed a very positive result considering their genetic diversity. Each cat bears a unique genetic background, and together they may better represent the heterogeneous human population. DNA electroporation was conducted without additional immune modulation aside from the addition of pfeGM-CSF, allowing room for improvement. In human HER2 transgenic mice, we found significant enhancement of HER2 vaccine response after regulatory T-cell depletion. It is possible to pursue various vaccine optimization and immune modulation strategies to improve vaccine response in cats.

Cancer vaccines may be more effectively advanced in veterinary medicine, as evidenced by the approval of a canine melanoma DNA vaccine (25) and by our findings. Because domestic animals share human dwellings and develop mammary carcinoma with similar frequency, biology, and histopathology to that of human breast cancer, it is beneficial to both cats and humans to further investigate vaccine responses in this companion animal species. Human trials testing novel vaccines are invariably initiated in patients who have failed other therapies, when their immune systems are compromised. In this study, we show that normal, healthy cats with functional immune systems develop promising responses to HER2 DNA electrovaccination. Given the aggressive nature of FMC and their propensity for HER2 expression, a preventive vaccine in cats merits consideration, particularly for breeder females. DNA is the most versatile vaccine formula and the design is limited only by one’s imagination. It is proposed that feline mammary tumors serve as a strong model for developing human cancer vaccines, warranting intensive investigation.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.
Studies supervision:
Analysis and interpretation of data (e.g., statistical analysis, biostatistics)
Development of methodology:
Conception and design:

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):

Writing, review, and/or revision of the manuscript:

Other (cloning of feline and 1stus americanus cDNA's used for construction of vectors and vaccines used in the study described in the article; STR analysis/authentication of feline cell lines used for construction of these vaccines, and for analysis of host immune responses to these vaccines):

References


Grant Support

Acknowledgments

The authors dedicate this work in memory of Dr. Marie Piechocki, who devoted her life to cancer research. The authors thank Dr. Elizabeth Dawe and Ms. Janine Matthei for their expert handling and care of the cats and Dr. Wendy Wiesend for her pathology expertise. The authors also thank the MCR, Genomics, Multidisciplinary Research, and AMTEC cores of the Karmanos Cancer Institute (P30CA22453) Special thanks to Lynn Marla, Colleen Bruning-Fann, Gail Chapman, Nancy Hendrick, Carol Logan, Linda Schroeder, and Colleen Seitz for the black bear harvest.

Received September 19, 2014; revised January 22, 2015; accepted February 14, 2015; published OnlineFirst February 23, 2015.

www.aacrjournals.org


Published OnlineFirst February 23, 2015; DOI: 10.1158/2326-6066.CIR-14-0175

HER2 DNA Vaccination of Domestic Cats


Induction of HER2 Immunity in Outbred Domestic Cats by DNA Electrovaccination

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Cancer Immunol Res  Published OnlineFirst February 23, 2015.

Updated version
Access the most recent version of this article at:
doi:10.1158/2326-6066.CIR-14-0175

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