Induction of HER2 Immunity in Outbred Domestic Cats by DNA Electrovaccination

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Abstract

Domestic cats share human living environment and genetic traits. They develop spontaneous feline mammary carcinoma (FMC) with similar histopathology as human breast cancer. HER2 and AKT phosphorylation was demonstrated in primary FMC by immunoblot, 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 bear 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 demonstrated 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 ~40% 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.
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 lab 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 fall 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). Since 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 where 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 amongst the incongruities between rodents and humans. The limitations of these models bring clarity to the need for intermediate animal systems that more closely resemble that in humans, have similar cancer etiology, express a comparable repertoire of TAAs and respond to the vaccine delivery approaches similar 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 residing in US households (8). Feline whole genome sequencing and single nucleotide
polymorphism 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 that in humans. Both 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, while 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 non-spayed 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 patients are treated with surgery, radiation and chemotherapy. Taken together, domestic felines represent a particularly suitable model for evaluating breast cancer vaccines.

DNA vaccines have advanced more expediently in veterinary than in human medicine. The first approved therapeutic cancer vaccine in any species was a plasmid DNA encoding heterologous (xenogeneic) human tyrosinase for treating 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 electrovaccination 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 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 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 aged 6 months-2 years were obtained from Liberty Research, Inc. (Liberty, NY). Animals were housed and maintained in the Department of Laboratory Animal Resource (DLAR) 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 care-taker 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 two feline patients treated at Oakland Veterinary Referral Services (OVRS) in Michigan with consent from the cat owners (Supplemental Table 1). 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

K248 cell line was established from a pulmonary metastasis of a Siamese cat that had a mammary carcinoma, and was provided by Dr. John Hilkens and the late Dr. Wim Misdorp at the Netherland Cancer Institute (40). Mammary carcinoma cell line K12 was from a 14 year old cat, and was established by Dr. William Hardy, Jr., and provided by Dr. Jaime Modiano of the University of Pennsylvania, PA (40). SKOV3 cells were purchased from the American Type
Culture Collection. MCF7 cells were obtained from Lisa Polin of the Karmanos Cancer Institute. All cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with fetal bovine sera, penicillin and streptomycin. The feline origin of K248 and K12 cells has been authenticated by short tandem repeat (STR) analysis of four loci (Supplemental Figure 1).

**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 histological-grade primary antibodies were applied according to 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 subcutaneously into SCID mice. Tumor explants were used as controls.

**DNA cloning and construction**

All cloning primer sequences are shown in Supplementary Table 2. Feline HER2 (ERBB2) cDNA 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 (feHER2) 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 pfeHER2 contains the signal peptide, and the extracellular and transmembrane domains of feline HER2. The pfeHER2-K contains a single nucleotide substitution in codon 141 (cag -> aag) of the extracellular domain 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 HER2 cDNA was similarly cloned from the liver tissue of a black bear. The cDNA sequence was submitted to Genbank (#JQ040508). DNA vaccine pbearHER2 encoding the signal peptide and the extracellular and transmembrane domains was constructed by PCR-based methods similar to that described for pfeHER2.

Feline GM-CSF cDNA 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, NM001009840, AF053007 and AF138140).

Generation of recombinant feline HER2 and human Fc fusion protein - feHER2-Fc

A plasmid encoding the secreted fusion protein of feline HER2 extracellular domain fused to human Ig Fc was constructed. Feline HER2 cDNA and the human Ig Fc domain were PCR amplified and fused by overlap-extension priming, giving a 2680 bp product, which was cloned into the HindIII and XbaI sites of pVax1. Murine 3T3 cells were transfected with this construct and the feHER2-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 Immuno). huHER2-Fc was purchased from Sino Biologicals.

Electrovaccination of mice and cats

Mice were injected with an admix of 50 μg each of vaccine plasmid and plasmid encoding murine GM-CSF (pmuGM-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 msec degenerating bipolar pulses of 100 V were administered at each site. Cats were injected with 1.5 mg each of HER2 vaccine plasmid and pFeGM-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 1.5 cm² caliper electrode (BTX).

Cell proliferation assay

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

Western blot analysis

Cells or tissues were lysed in a non-ionic 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). Ten μg protein was boiled in Laemmli buffer, separated with 8% SDS-polyacrylamide (PAGE) gel and transferred onto PVDF 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 Biotech). 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, Weissman Institute, Israel). mAb to human EGFR (528, Santa Cruz Biotech), 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 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 (SantaCruz). 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 Roswell Park Memorial Institute medium supplemented with fetal bovine sera, penicillin/streptomycin. Feline PBMCs were supplemented with 0.5 ng/mL feline IL2 (R&D Systems). Cells were plated at 2x10^5/well in round bottom 96-well plates and cultured with 10 μg/mL feHER2Fc (3T3 supernatant-equivalent as described above), huHER2Fc, human IgG control (Jackson Immunolabs) 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 prior to detection and enumeration as previously described (44) or per manufacturer protocol. Visualized cytokine spots were enumerated using the ImmunoSpot analyzer (CTL, Shaker Heights, OH) and expressed as the number of cytokine-producing cells per 10^6 splenocytes or PBMCs.
Results

Expression of HER2 in feline mammary carcinoma (FMC)

Expression of \textit{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 (Figure 1A). MHC I expression was also detected with mAb W6/32 to a constant region of 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 (Supplemental Table 1) was detected by immunohistochemical (IHC) staining (Figure 1B). Membrane staining of HER2 was detected in all three primary FMC samples and in K248 explant, consistent with membrane staining of K248 cells by flow cytometry (Figure 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, Supplemental Figure 2). Although cross-reactivity with feline antigens by all antibodies in this array was not verified, AKT phosphorylation (S473) was elevated in 3/3 FMC tissue samples CSU-133, 418, and 1646, compared to 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 (Figure 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 if ERRB RTK signaling is required for FMC cell proliferation, FMC cell lines K12 and K248 were cultured with or without ERRB family tyrosine kinase inhibitors gefitinib or lapatinib (Figure 2B). Both K12 and K248 exhibited dose-dependent inhibition of cell proliferation. SKOV3 and MCF7 cells were the positive and negative control, respectively. Therefore, FMC express functional tyrosine kinase receptors which trigger downstream signaling and cell proliferation.

**Cloning and characterization of feline HER2**

Feline HER2 (*ERBB2*) cDNA cloned from K12, K248 and normal ovary showed identical sequences (GenBank Accession JN990983). The amino acid (a.a.) translation of the full-length feline HER2 shared 93% sequence identity with human HER2 (Figure 3A and Supplemental Figure 3). 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 (ECTM) 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) (46-49) (Figure 3B). Feline HER2 was recognized by all five mAbs. The closely related black bear HER2 was recognized by four 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 4 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) containing human ERBB2
extracellular domains (ECD) 1/2, rat neu ECD 3/4 and neu transmembrane domain induced both humoral and cellular immunity in HER2 Tg mice (4). Based on these findings, heterologous bear, human and rat ERBB2 genes sharing high levels of sequence identity with feline ERBB2, and were tested as candidate vaccines. To evaluate if minimal alteration could be engineered to afford immunogenicity while preserving feline HER2 epitopes, a single amino acid, glutamine-141 in domain 1 of the feline HER2 ECD, was replaced with lysine to generate \textit{feHER2-K}.

Supplemental Figure 4A/B show the predicted 3D structure of \textit{feHER2-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 2.1.1 software; Tripos), where the electropositive side chain of K is indicated by red and Q is shown in blue (Supplemental Figure 4C).

**Immunogenicity of \textit{feHER2-K}**

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

Immunogenicity of \textit{feHER2} and \textit{feHER2-K} was initially tested in BALB/c mice by electrovaccination with an admix of \textit{pfeHER2}, \textit{pfeHer2-K} or control \textit{phuHER2} (encoding human HER2 ECTM) together with \textit{pmuGM-CSF} encoding murine GM-CSF (4, 6, 42,50). After 2x immunization, 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 (Figure 4B). Expression of \textit{feHER2}, \textit{feHer2-K} or \textit{huHER2} on individually transfected 3T3 cells was comparable as verified with mAb TA-1 binding at 2 different concentrations (Supplemental
Figure 5A). peHER2 or peHER2-K antibodies are highly cross-reactive, indicating the recognition of dominant foreign epitopes on feline HER2 (Figure 4B, left and middle panels). The Q141K substitution appears to create only subtle changes which 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 un-transfected 3T3 cells (Supplemental Figure 5B), supporting the HER2 specificity of the immune sera.

To measure T-cell response, a feline HER2 extracellular domain (ECD) and human Ig Fc fusion protein, feHER2-Fc, was generated as the test antigen and verified by Western blot (Figure 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 peHER2, peHER2-K or phuHER2, showing cross-reactivity between feline and human HER2 antigen (Figure 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 (pfeGM-CSF). Generation of recombinant GM-CSF in the supernatant of transfected 3T3 cells was verified by Western blot (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 12-24 months of age were injected
with pE2Neu and pfeGM-CSF in three legs in the biceps femoralis or quadriceps followed by electroporation. Vaccination was administered 4x 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 two cats and 1:100,000 in the other (Figure 5A, left panel). Rat neu binding Ab ranged from 1:25,000 to 1:100,000 (Figure 5A, middle panel). Vaccination with pE2Neu induced antibody that also recognized 3T3 cells expressing WT feline HER2 (Figure 5A, right panel), showing reactivity to self-HER2. Neither pre- nor post-4th-vaccination feline serum stained un-transfected 3T3 cells, illustrating the HER2/neu-specific reactivity of the immune sera (Supplemental Figure 6A).

**Immune response to novel HER2 DNA vaccines**

A panel of HER2 vaccines was tested in fifteen additional healthy cats between 5-8 months of age. Cats were electrovaccinated 4x with pfeHER2, pfeHER2-K, pbearHER2, pE2Neu, or an admix vaccine of pfeHER2-K and pE2Neu. As observed previously, the six 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 (Figure 5B, left panel). pbearHER2 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 (Figure 5B, middle panel). A prominent response (~1:120,000) was induced by pfeHER2-K+pE2Neu. Individually, pfeHER2-K, pE2Neu or pbearHER2 induced moderate titers averaging ~1:6,000, a 10-20 fold reduction from the admix vaccine, suggesting a synergistic or adjuvant effect of the heterologous pE2Neu. pfeHER2 vaccination did not generate a significant antibody response to feHER2-K.
Binding to 3T3 cells expressing WT feline HER2 (3T3/feHER2) was detected between 1:1,600-1:3,200 with immune sera from pE2Neu, pfeHER2-K+pE2Neu, or pbearHER2 vaccinated cats (Figure 5B, right panel) and may be a result of antigenic cross-reactivity between heterologous HER2 and feline HER2. pfeHER2 and pfeHER2-K immune sera showed negligible binding to 3T3/feHER2. The specificity of HER2 antigen recognition was validated by the absence of binding to un-transfected 3T3 cells after 4x vaccination (Supplemental Figure 6B).

The reactivity of immune sera was further tested at 1:50 - 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 (Figure 5C). pfeHer2 immune sera showed little binding to any of the test cells. pfeHER2-K immune sera also showed little or low level binding to 3T3/feHER2, but significant binding to K12 and K248 cells (Figure 5C, middle and right panel). Although other immune sera recognized 3T3/feHER2, they reacted minimally with K12 or K248 cells. These findings may suggest that feHER2-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 three days before IFNγ ELISPOT analysis (Figure 6A). The 6 cats receiving pE2Neu with or without pfeHER2-K responded to human HER2 at 130-750 SFU/10⁶ PBMCs, validating the effectiveness of DNA electrovaccination (Figure 6A). pbearHER2 immune T cells also cross-reacted with human HER2, producing an average of ~160 SFU/10⁶ PBMCs. Vaccination with pfeHER2-K alone did not induce T cells that recognized human HER2.

IFNγ T-cell response to feHER2-Fc was measured to evaluate reactivity to self-HER2
(Figure 6B). Of the 10 cats evaluated, three produced significant feHER2-specific T-cell responses, with one cat each from the pfeHER2-K (~100 SFU per 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 five cats that received pfeHER2-K or pfeHER2-K+pE2Neu (Figure 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 total of 40% T-cell response rate to self-HER2 was achieved in healthy cats. Those cats initially receiving pE2Neu or pbearHER2 were boosted 3x with pfeHER2-K (Figure 6D/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 6-12 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 amino acid 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 2/5 healthy cats, vaccination with pE2Neu or pbearHER2 overcame immune tolerance to trigger T-cell responses to self-feHER2. Vaccination with pfeHER2-K also induced feHER2-specific T-cell responses in 1 of 3 cats. Additional studies will be necessary to validate and enhance T-cell activation by feline HER2 vaccines. Antibody binding to 3T3/feHER2 was detected in cats vaccinated with heterologous HER2. Although pfeHER2-K immune sera were the least effective in binding 3T3/feHER2, these immune sera recognized unique epitopes on FMC K12 and K248. Taken together, outbred cats represent a new model system for evaluating novel 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.

feHER2-K immune sera appear to bind epitopes on 3T3/feHER2-K as well as on K12 and K248 FMC, but only minimally to 3T3/feHER2. Although other interpretations may exist, we propose the following scenario: feHER2-K recapitulates a cryptic HER2 epitope naturally displayed when WT feHER2 is heterodimerized or associated with other ERBB family members. Cats immunized with heterologous pE2Neu or pbearHER2 produced antibodies that recognize dominant epitopes on WT feHER2 as well as 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 HER2 epitope recognition was made possible by vaccinating genetically unmanipulated cats that naturally express HER1, HER2, and HER3. It will be important to further evaluate immune sera from feline HER2 vaccines to better define the epitopes they recognize and those epitopes important for controlling signaling and tumor growth. Rationally designed HER2 DNA vaccines, engineered with modifications to specific domains, may help bring clarity.

Our previous study showed consistent responses to HER2 DNA electrovaccination in BALB/c 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 30-40% 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. Since domestic animals share human dwellings and develop mammary carcinoma with similar frequency, biology and histopathology as 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 the current 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.

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References


Figure Legends

**Figure 1. Expression of HER2 in feline mammary carcinoma.** (A) Flow cytometric analysis of cell surface EGFR (HER1), HER2, HER3 and MHCI 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 40x magnification.

**Figure 2. RTK activity in FMC.** (A) Western blot analysis of total HER2, pHER2 (Y1248), total Akt and pAkt (S473) in primary FMC samples. Feline PBMCs were the negative control. (B) Sensitivity of FMC cell lines to receptor tyrosine kinase inhibitors. Cells were cultured in gefitinib or lapatinib for 48 h and % proliferative cells was measured with Alamar Blue by comparison to untreated controls. *p<0.001 two-way ANOVA with Dunnet's posttest.

**Figure 3. HER2 sequences and epitopes.** (A) BLAST analysis of full-length HER2 amino acid sequence identity. (B) Schematic of binding by 4 mAbs to human HER2 (TA-1, N29, N12, 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.

**Figure 4. Expression and immunogenicity of feHER2-K.** (A) Binding of 3T3/feHER2-K by mAb to HER2/neu. Staining and analysis 3T3/feHER2-K were conducted as in Figure 3B. (B) Antibody responses to pfeHER2, pfeHER2-K and phuHER2 in BALB/c mice before (Pre) and two weeks after 1x and 2x vaccination given in 2-week intervals. Horizontal lines depict the
mean for each group (*p<0.05, **p<0.005 one-way ANOVA with Tukey’s posttest). (C)
Generation of recombinant feline HER2 and human Ig Fc fusion protein (feHER2-Fc) and
immunoblot analysis. (D) T-cell response to feline and human HER2 in 2x vaccinated mouse
splenocytes (n=4) or naïve mouse splenocytes (n=3) as measured by ELISPOT in triplicates
using 10 µg recombinant antigen (*p<0.05, **p<0.01 two-way ANOVA with Dunnet’s posttest).

**Figure 5. Induction of HER2 antibody in cats.** (A) Feline anti-huHER2 (left), rat neu (middle)
or feHER2 (right) IgG titer induced by pE2Neu and pfeGM-CSF vaccination. Serum samples
were collected just before and 2 weeks after each of 4 vaccinations given in 3-week intervals.
Antigen binding was analyzed by flow cytometry using transfected 3T3 cells. (B) Feline anti-
huHER2 (left), anti-feHER2-K (middle) or anti-feHER2 (right) IgG titer induced by the indicated
vaccine. Serum samples were collected just before and 2 weeks after each of 4 vaccinations
given in 3-week intervals. Antigen binding was analyzed by flow cytometry using transfected
3T3 cells. (C) Binding of immune sera to 3T3/feHER2 (left), K12 (middle) or K248 (right) cells
prior to (shaded histogram) and two weeks after the 4th vaccination (open histogram). Each
overlayed histogram represents an individual cat.

**Figure 6. Induction of HER2-specific T cells in cats.** PBMCs were harvested after 4x
vaccination and stimulated with 10 µg/mL (A) rhuHER2-huFc, (B) rfeHER2-huFc or human IgG
control. Results are presented as IFNγ spot forming units (SFU)/10^6 PBMCs. (C) Feline HER2
specific T-cell response after a total of 7 vaccinations with pfeHER2-K with or without pE2Neu.
(D) Human or (E) feline HER2-specific T-cell response induced by pE2Neu or pbearHER2
followed by pfeHER2-K. *p<0.05, **p<0.01 Mann-Whitney test.
Figure 1

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**Sensitivity to Gefitinib**

**Sensitivity to Lapatinib**

- K12
- K248
- SKOV3
- MCF7
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Full-length HER2 BLAST analysis [%Identities / %Positives / %Gaps]

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B

Subdomain I Subdomain II Subdomain III Subdomain IV TM

TA-1 7.16.4 N29

N12 Trastuzumab

Feline HER2

Bear HER2

Human HER2

Rat neu
Figure 4
Cancer Immunology Research

Induction of HER2 Immunity in Outbred Domestic Cats by DNA Electrovaccination

Heather M Gibson, Jesse Veenstra, Richard F. Jones, et al.

Cancer Immunol Res  Published OnlineFirst February 23, 2015.

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