HER2-Targeted Therapy Reduces Incidence and Progression of Midlife Mammary Tumors in Female Murine Mammary Tumor Virus huHER2-Transgenic Mice

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ABSTRACT

Purpose: This study examined the effectiveness of early and prolonged mu4D5 (the murine form of trastuzumab/Herceptin) treatment in transgenic mice that overexpress human HER2 (huHER2), under the murine mammary tumor virus promoter, as a model of huHER2-overexpressing breast cancer.

Experimental Design: Mice were randomly assigned to one of three treatment groups and received i.p. injections from 17 weeks of age until either 52 weeks of age or morbidity. Fourteen mice received 100 mg/kg mu4D5, 14 mice received 100 mg/kg antiherpes simplex virus glycoprotein D control antibody, and 11 mice received a diluent control.

Results: High levels of huHER2 expression were detectable in mammary glands of young virgin founder mice. Mammary adenocarcinomas were frequently found in female founders and progeny at an average age of 28 weeks, with some progressing to metastatic disease. The incidence of mammary tumors was significantly reduced, and tumor growth inhibition was observed in mice receiving mu4D5 compared with control mice. In addition, Harderian gland neoplasms, highly associated with overexpression of huHER2 in this transgenic line, were entirely absent in the mu4D5 treatment group, indicating down-regulation of huHER2 in vivo activity.

Conclusions: Early intervention with mu4D5 was of benefit in our transgenic mice at high risk for developing huHER2-overexpressing breast cancer. This study suggests a potential benefit of early treatment with Herceptin in HER2-positive primary breast cancer.

INTRODUCTION

Amplification and overexpression of HER2 (ErbB2), a 185-kDa type 1 receptor tyrosine kinase belonging to the epidermal growth factor receptor (ErbB) family, is found in ~30% of human breast cancers and is associated with refractory responsiveness to chemotherapy and overall poor prognosis (1, 2). HER2 overexpression is thought to lead to ligand-independent association/dimerization of HER2 receptors, resulting in constitutive activation and signaling through the potent ErbB2 tyrosine kinase pathway (3). Overexpression of HER2 has also been implicated in tumor progression of other human epithelial cancers, such as ovarian, gastric, pancreatic, and non-small cell lung cancer (4).

Transgenic mouse models have proved useful in the characterization of oncogene activities in specific tissues, including those of neu, the rodent form of HER2/ErbB2 (5–9). Previous mouse models of neu overexpression, targeted to the mammary gland under the direction of the murine mammary tumor virus (MMTV) promoter, have used either the normal (10) or the activated (mutated) form of neu, characterized by an amino acid change at position 664 in the transmembrane domain from valine to glutamic acid (8, 11, 12). In the study by Muller et al. (8), all multiparous female transgenics harboring the activated form of neu showed single-step induction of polyclonal mammary adenocarcinomas by 78–95 days of age, with occasional progression to pulmonary metastasis. Interestingly, when Bouchar et al. (12) constructed activated neu-transgenic mice using a slightly different transgene design, in contrast to the results of Muller et al. (8), female transgenics demonstrated a less robust, stochastic induction of mammary adenocarcinomas, with a latency of 5–10 months. Likewise, when normal neu was ectopically expressed in MMTV-transgenic mice using the original construct from Muller et al. (Ref. 8; but lacking the activating transmembrane amino acid change), fewer mammary tumors developed and a significantly longer latency (120–337 versus 78–95 days) was necessary for tumor development. The characteristics of focal tumor formation and variable, yet generally extensive latencies, imply that additional genetic changes beyond neu overexpression are likely required for mammary tissue transformation leading to tumor formation (10).

Similar models using the human HER2/ErbB2 (huHER2) gene have proved problematic. Suda et al. (13) observed adenocarcinomas of lung and Harderian gland but not mammary gland, as well as a high incidence of B-cell lymphomas, with all neoplasms occurring after a relatively long latency. However, because of the inherent limitations of the rodent neu-transgenic models, which, although useful for the study of basic HER2 biology and of nonspecific therapies, are not suitable for examining therapeutics specifically targeted to the human form of HER2, we have developed a reliable mouse model of huHER2-overexpressing breast cancer. By creating transgenic mice with...
the normal human HER2 gene under the control of the MMTV promoter using an optimized transgene construct, our mouse model of huHER2-overexpressing breast cancer can be used for testing targeted therapeutics such as trastuzumab (Herceptin).

The humanized version of the mu4D5 antibody, Herceptin, specifically directed against human HER2, is efficacious and well tolerated in the treatment of HER2-positive breast cancer patients with metastatic disease, and based on survival data from such patients, it is predicted that treatment with Herceptin will also prove to have a positive effect on patients with primary breast cancer (14). To further examine the potential benefits of Herceptin treatment, we decided to use our transgenic model to test the effect of early and prolonged mu4D5 treatment (the murine form of trastuzumab was used to avoid a mouse anti-human antibody response with long-term therapy), using females at high risk of developing huHER2-overexpressing breast cancer (15–17).

MATERIALS AND METHODS

**MMTV.f.huHER2 Construction Design and Preparation for Microinjection.** The vector backbone containing the mammary tumor LTR as previously described (9) was modified by introducing linkers (PvuI, ApaLI, AsnI, and BspH1) 5′ of the MMTV promoter and 3′ of human growth hormone exons 4 and 5, the 3′-untranslated region, and the polyA region, to allow for purification of the 6569-bp fragment for microinjection. The huHER2 cDNA, after *Nco*I digestion (CCATGG) and religation to remove the 26-bp fragment, which includes an upstream ATG removed to prevent *in vitro* inhibition of translation of the full-length *huHER2* (18), was then introduced into the multiple cloning site via a *Khol* restriction cut and subsequent ligation. The Promega pCI chimeric intron (E1731) was introduced 3′ of the MMTV promoter and 5′ of the *huHER2* cDNA via a *Hind*III site (Fig. 1A). The transgene fragment was then isolated from the plasmid backbone by a *PvuI* digestion. The resulting 6569-bp fragment was isolated by agarose gel purification via a Qiaquick kit (Qiagen Corp.) and diluted to 2 ng/μl in 20-μM filtered TE [10 mm Tris and 1 mm EDTA (pH 7.5)] in preparation for microinjection. All animal care was in accordance with NIH guidelines.

**Production and Identification of huHER2-Positive Transgenic Mice.** The FVB inbred mouse strain was used because of its known suitability for transgenic experimentation and genetic analysis (19). One-cell FVB embryos were collected, microinjected in several rounds at either DNX Transgenics or at Genentech, Inc., and transferred into pseudopregnant CD-1 females, as described previously (20). Nine-day-old founder mice were ascertained to be transgenic by PCR using DNaseasy (Qiagen Corp.)-prepared tail DNA and a primer set specific to human growth hormone exons 4 and 5 (forward-5′-CACCACTTAAAGGACCTAGAGGAAGGC-3′ and reverse-5′-CAAGGCCAGGAGAGGCACTGGGGAG-3′).

Transgenic founders were maintained, when not breeding, on the AIN 76A-Teklad-purified diet, with casein for protein, cornstarch and sucrose for carbohydrates, corn oil for fat, and purified cellulose for fiber, a diet shown to enhance mammary tumor formation in a previously published study (21).

The transgenic line MMTV.f.huHER2 #5 (Fo5) was used for this study.

**Surgical Mastectomy Technique.** At 13 weeks of age, transgenic female mice were anesthetized with isoflurane. The ventral surface of the abdomen was shaved and prepared with Betadine and 70% isopropyl alcohol. Using aseptic technique throughout, an incision was made lateral to the midline near the no. 4 inguinal nipple. Skin was freed from the abdominal wall, and the mammary fat pad was excised from the skin, followed by closure of the incision with silk suture. Females were closely observed and received 1–2 mg/ml Tylenol elixir in their drinking water for 7 days postsurgery. One-half of the mammary sample was flash frozen in liquid nitrogen for Taqman expression analysis; one-half of the sample was fixed in 10% formalin for sectioning, histology, and immunohistochemistry.
Mammary Tumor Monitoring. Characteristically, mammary tumors were initially discovered by palpation at an average size of 1 mm³. Upon discovery, tumor measurements were collected twice weekly. Primary mammary tumors were monitored and photographed, and routine tumor resection was performed whereby mammary tumors were surgically removed upon reaching ~1.5 cm in diameter, generally within 4–6 weeks of discovery. Photographs and tumor samples were collected at each surgical intervention point. Postoperatively, transgenic females were observed visually and mammary glands palpated for the appearance of a second tumor (de novo or recurrent). Mice were also examined throughout the study for Harderian gland enlargement.

Tumor Removal Survival Surgery Technique. Surgical preparation was similar to that above. Surface area associated with tumor was shaved and prepared with Betadine and 70% isopropyl alcohol. Using aseptic technique throughout, an incision was made lateral to the midline tumor periphery. Tumor was freed from the skin and abdominal wall; a sample of adjacent normal mammary tissue was excised along with the tumor. This was followed by closure of the incision with silk suture. Females were closely observed and received 1–2 mg/ml Tylenol elixir in their drinking water for 7 days postsurgery. One-third of the tumor sample was flash frozen in liquid nitrogen for Taqman expression analysis; two-thirds of the sample was fixed in 10% formalin for sectioning, histology, and immunohistochemistry.

Preparation of Mouse Tissues for Histopathology Analysis. At necropsy, tissues were placed into 10% neutral-buffered formalin for overnight fixation and then transferred into 70% ethanol before processing and paraffin embedding. For routine histopathological analysis, 5–μm thick paraffin sections were stained with H&E.

Immunohistochemical Analysis of huHER2 Expression in Positive Transgenic Mice. Immunohistochemical analysis of huHER2 protein expression was performed on paraffin sections using the humanized version of mu4DS, Herceptin, as the primary antibody at a concentration of 5 μg/ml. This antibody does not recognize murine HER2; the humanized instead of the original murine version was chosen to avoid background staining with the secondary (antihuman immunoglobulin) antibody in the presence of endogenous murine immunoglobulin. Incubation with primary antibody was performed at 4°C overnight after antigen retrieval with pepsin (0.4% in 0.1 N HCl at 37°C for 5 min). Specifically bound primary antibody was visualized with a biotinylated goat antihuman IgG (30 min at room temperature) followed by ABC Elite reagent (30 min at room temperature; Vector Laboratories, Burlingame, CA) and 3,3′-diaminobenzidine.

Serum ELISA for Shed huHER2 Extracellular Domain (ECD). Serum was collected from positive transgenic mice at 13 weeks of age and an ELISA (assay range of 2–0.03 ng/ml) was run to detect the presence of shed huHER2 ECD. The assay used goat anti-HER2 diluted to 0.835 μg/ml to coat NUNC MaxiSorp 96-well plates and followed a procedure similar to that described previously (22). Recombinant huHER2 ECD (Genentech, Inc., South San Francisco, CA) was used as the standard and was diluted to 4 ng/ml following by additional 1:2 dilutions in assay buffer [PBS/0.5% BSA, 0.05% Tween 20/10 ppm of Proclin 300/0.2% BGG/0.25% CHAPS/0.15 m NaCl/5 mm of EDTA (pH 7.4)]. Similarly, mouse serum samples were initially diluted to 1:50, followed by additional 1:2 dilutions in assay buffer. Plates containing sample and standard were incubated at 4°C overnight, followed by six washes with wash buffer (PBS/0.05% Tween 20/0.01% thimerosal); biotin-conjugated rabbit anti-HER2 was then added (100 μl/well) at a concentration optimized for each batch of conjugated antibody. Plates were then incubated for an additional 2 h and washed six times. Amxest-strapavidin-horseradish peroxidase (Amersham Pharmacia Biotech, Piscataway, NJ) was then added at a dilution of 1:5000 in assay buffer for 1 h, followed again by washing. One-hundred μl of tetramethyl benzidine substrate (Kirkegard and Perry Laboratories, Gaithersburg, MD) were subsequently added to each well and color allowed to develop for 10–15 min. The reaction was stopped with the addition of 100 μl of 1 m phosphoric acid. Plates were read at a wavelength of 450 nm with a 620 reference using a microplate reader ( Molecular Devices, Sunnyvale, CA, or Titertek, Huntsville, AL). The concentration of huHER2 ECD in the serum samples was extrapolated from a four-parameter fit of the standard curve. The intra- and interassay coefficients of variation were calculated by ANOVA using the Statview program.

Taqman Reverse Transcription (RT)-PCR for Expression Analysis of huHER2 mRNA from Transgenic Mice. Message levels were quantified by real-time RT-PCR Taqman as described previously (23). RNA from various tissues and tumors was analyzed for the gene of interest, huHER2, and the housekeeping gene mouse ribosomal protein L-19 (RPL19). In Fig. 4, relative gene expression was calculated using the standard curve method (Applied Bio Systems, user bulletin no. 2; ABI PRISM 7700 sequence detection system). Briefly, RNA from MCF-7 cells, a low-level huHER2-expressing breast cancer cell line from the American Type Culture Collection, was serially diluted in water (0.8–500 ng) and was used to construct the standard curve for both huHER2 and RPL19. Relative gene expression was calculated for any unknown sample by first solving for the mass of unknown RNA via the standard curve. Next, the amount of huHER2 RNA was divided by the amount of RPL19 RNA to determine the normalized amount of huHER2. Four female huHER2-positive and four female huHER2-negative transgenic mice, all 2.5 months of age, were used to measure human HER2 expression in this study. In Fig. 5F, relative gene expression was calculated for samples using the comparative threshold cycle (Ct) method (Applied Biosystems, ABI PRISM 7700 sequence detection system). The primer set used to quantitate transgenic huHER2 message was specific to exon 27 of the huHER2 gene. The sequence of the primer/probe sets were as follows: (a) HER2, human HER2-forward 5′-TCTGGACGTGCCAGTGGTA-3′; human HER2-reverse 5′-TGTCCTCCTGAGGACACT-CA-3′; and human HER2-probe 5′-CAGAAGGCCAAGTCCGACAGAAG-3′; and (b) RPL19, mouse RPL19-forward 5′-GGTCAGGCCAGGCTCTTG-3′; mouse RPL19-reverse 5′-GGCCATCTCCATGAGGAGCACA-3′; and mouse RPL19-probe 5′-CACAAGCTGAACGAG-CAAGGC-3′.

RNA isolation and Sequence Analysis of huHER2 in Transgenic Tissues. Total RNA was isolated from various tissues and tumors from huHER2-transgenic and wild-type mice.
using a Qiagen RNAeasy kit (catalogue no. 74181). RT-PCR was performed to reverse transcribe and amplify the huHER2 gene using a Qiagen One-Step RT-PCR kit (catalogue no. 210210). PCR products were run on agarose gels; the expected bands were then isolated from the gel. The DNA was extracted and sequenced to determine whether mutations were present in the juxtamembranous or transmembrane domains. Sequences of the primers used for RT-PCR and sequencing of amino acids 540 to 740 were as follows: human HER2-forward 5′-CCAGATGCTCAGTGACCTGT-3′; and human HER2-reverse 5′-GATCCAGATGCCCTTGAGACT-3′.

**FISH (Fluorescence in Situ Hybridization) Detection of the Transgenic Integration Site in the Fo5-Transgenic Line.** One male spleen from the Fo5-transgenic line was sent to SeeDNA Biotech, Inc. (Windsor, Ontario, Canada). Chromosome slide preparation: lymphocytes were isolated from the spleen upon receipt and cultured in RPMI 1640 supplemented with 15% FCS, 3 μg/ml concanavalin A, 10 μg/ml lipopolysaccharide, and 5 × 10⁻⁵ M mercaptoethanol. After 44 h, the cultured lymphocytes were treated with 0.18 mg/ml bromodeoxyuridine for an additional 14 h. The synchronized cells were washed and recultured at 37°C for 4 h in α-MEM with thymidine (2.5 μg/ml). Chromosome slides were made by conventional method preparation (hypotonic treatment, fixation, and air-dried). Probe labeling and FISH detection: the 6569-bp transgene fragment used for microinjection was used as the probe for FISH analysis, with a detection efficiency of 95% (among 100 checked mitotic figures, 95 showed hybridization signals on the one specific chromosome with no other positive locus detectable). The probe was biotinylated with dATP using the BRL BioNick labeling kit (15°C, 1 h; Ref. 24), and FISH analysis was performed as described previously (24, 25). FISH signals and the 4',6-diamidino-2-phenylindole banding pattern were recorded separately by taking photographs; the assignment of the FISH mapping data with the chromosomal bands was achieved by superimposition of FISH signals with 4',6-diamidino-2-phenylindole banded chromosomes.

**Treatment of Transgenic Cohorts: Anti-huHER2 mu4D5, antiherspes simplex virus glycoprotein D (Anti-gD), and Diluent Control.** Both mu4D5 (anti-huHER2) and anti-gD control antibody 3C8 clone 1766 were subtype IgG1. Fourteen mice received mu4D5 formulated and diluted to 1.28 mg/ml in 5 mM sodium acetate, 150 mM NaCl, 0.01% Tween 20 (pH 5.0). An additional 14 mice received anti-gD formulated and diluted to 12.9 mg/ml in PBS. Eleven mice were assigned to an additional control group and received a diluent control, either 5 mM sodium acetate, 150 mM NaCl, 0.01% Tween 20 (pH 5.0), or PBS. Appropriate volumes (from 150 to 200 μl based on animal weight) were injected i.p. weekly using 1 ml tuberculin syringes to deliver 100 mg/kg antibody/mouse/injection. Diluents were similarly delivered based on corresponding volumes/body weight. Mice were injected weekly from 17 weeks of age until either the development of a second 1.5-cm³ tumor (de novo or recurrent) or morbidity. The study was terminated at 52 weeks of age.

**HER2 ECD Binding ELISA to Determine Circulating Levels of mu4D5.** Serum was collected monthly and an ELISA run to determine the circulating levels of mu4D5 using a procedure similar to that described previously (26). NUNC MaxiSorp 96-well plates were coated with 0.5 μg/ml of purified recombinant huHER2 ECD (Genentech, Inc.). Standards (mu4D5) and mouse serum samples were diluted in assay buffer [PBS,0.5% BSA, 0.05% Tween 20/10 ppm of Proclin 300,00.2% BGG/0.25% CHAPS/0.15 mM NaCl/5 mM of EDTA (pH 7.4)] and 100 μl/well was added to the coated plates. Plates were incubated for 2 h at room temperature with gentle agitation followed by six washes with wash buffer (PBS,0.05% Tween 200,0.1% thimerasol). Horseradish peroxidase-conjugated goat anti-mu-Fc (Jackson ImmunoResearch Laboratory, West Grove, PA) was added (100 μl/well) and incubated for 2 h at room temperature. Plates were then washed to remove any unbound conjugate; 100 μl/well tetramethyl benzidine (two-component substrate kit from Kirkegard and Perry Laboratories) were added and incubated for 15 min. The color reaction was stopped by the addition of 100 μl of 1 M H₃PO₄, and the resulting absorbance was read at 490 nm on a Vmax plate reader (Molecular Devices, Menlo Park, CA). A standard curve was generated by plotting absorbance versus ECD concentration. Serum sample concentrations were obtained by interpolation of absorbance measurements on the standard curve.

**Statistical Analysis of Control and mu4D5 Treatment Data Points.** For each group of mice, the Kaplan-Meier estimate of disease-free survival was calculated (27).

**RESULTS**

**Characterization of the MMTV.huHER2 #5 (Fo5)-Transgenic Line.** At 13 weeks of age, serum from Fo5 was collected and found to contain 1.5 ng/ml shed huHER2 ECD based on ELISA analysis. No shed ECD was detected in any nontransgenic littermates in the Fo5 line. In addition, histological analysis and IHC were performed on mastectomy tissue from Fo5, revealing strong huHER2 expression in the epithelium of the mammary gland (Fig. 2, A and B).

To further characterize the Fo5 line, the transgene was determined to have integrated at a single site on murine chromosome 6, region D, an unknown locus site, by FISH analysis. Intensity of probing and quantitative PCR analysis suggested a transgene copy number of 30–50 (Fig. 2D–F).

The Fo5-transgenic female was first bred with a p53 heterozygous male (129/BL/6 × FVB, F1), delivering four litters with the expected transgene transmission rate of 50%. The p53 heterozygous background was used in the aim of enhancing mammary tumor formation and potentially decreasing tumor latency in our model. Seventy-eight percent of female positive transgenics from the Fo5 line, on a p53 heterozygous background was used in the aim of enhancing mammary tumor formation and potentially decreasing tumor latency in our model. Seventy-eight percent of female positive transgenics from the Fo5 line, on a p53 heterozygous background developed asynchronous mammary tumors with an average latency of 28.2 weeks. Interestingly, 76% of female positive transgenics from the Fo5 line developed asynchronous mammary tumors with an average latency of 28.2 weeks (the earliest onset noted was 23 weeks) on a p53 wild-type background. Hence, the use of the p53 heterozygous background offered no significant advantage in our model, confirming a recent published study in which the loss of p53 had little effect on the rate or stochastic appearance of mammary tumors in another MMTV-activated c-neu-transgenic model (28). Breeding was therefore continued on the original inbred FVB background strain after only two generations.
At 23.7 weeks of age, the Fo5 founder mouse was observed, during weekly mammary gland palpation, to have a primary mammary tumor of \( \frac{1}{11011} \) mm\(^3\) that ultimately grew to span two adjacent breasts. Histologically, the tumor demonstrated features of an adenocarcinoma of the breast with areas of solid, tubular, and papillary growth, cellular pleomorphism, and mitotic activity (Fig. 3A); immunohistochemistry revealed strong but somewhat heterogeneous membranous staining for huHER2 in tumor tissue (Fig. 3B) and strong staining of adjacent, nonneoplastic mammary ducts (data not shown). Fig. 3C illustrates a primary breast tumor just before tumor removal survival surgery.

Similarly, primary mammary tumors have consistently developed in a stochastic manner in Fo5 female-transgenic progeny. All mammary tumors have been classified as rapidly growing adenocarcinomas. Pulmonary metastases have been noted in 23% of the Fo5 female transgenics that have undergone this surgical intervention procedure (Fig. 3D). The histological features of the pulmonary tumor lesions that have undergone this surgical intervention procedure (Fig. 3E). As with the original Fo5 mammary tumor, the metastatic lesions were also characterized by a high level of huHER2 expression (Fig. 3F) comparable with the strong staining of the primary mammary tumor tissue of origin (Fig. 3, G and H). Transgenic females from the Fo5 line, currently at generation F11, continue to reliably exhibit the described mammary tumor phenotype.

Although the MMTV promoter is noted for its targeted expression to the mammary gland, integration site effects are known to play a role in expression pattern and expression level (9). To ascertain the expression pattern of huHER2 in other tissues in the Fo5 line, selected tissues from four female transgenic mice were flash frozen in liquid nitrogen for total RNA isolation. Interestingly, huHER2 expression was found in numerous tissues as well as mammary gland, albeit at variable levels, when Taqman analysis with a probe to huHER2 was used (Fig. 4). Taqman results were also verified by immunohistochemistry in several positive tissues such as salivary gland, pancreas, and bronchial epithelium, with focal huHER2 expression patterns often noted (data not shown).

An essentially identical pattern of expression of huHER2 was observed in Fo5 male transgenics. In addition, positive immunohistochemical staining for huHER2 was noted in rare prostatic epithelial cells (data not shown). No tumors have yet been observed in transgenic males, regardless of age or founder
line. Interestingly, with levels of huHER2 expression noticeably elevated in most tissues in both sexes in the Fo5 line, all tissues appeared architecturally normal and invasive adenocarcinomas have only been seen in the female mammary gland, although Harderian gland enlargement was a frequent finding, as described below.

The Harderian gland, a sebaceous gland in mice that excretes fluid facilitating movement of the third eyelid, is a tissue also targeted by the MMTV promoter and was confirmed to highly express huHER2 in this founder line. Enlargement of the Harderian gland was frequently observed in the positive transgenic mice from the Fo5 line, particularly in aging mice who had yet to develop mammary tumors. This often led to the need to sacrifice these animals because of progressive exophthalmus (Fig. 5, A and B). In this study, we observed no such Harderian gland enlargement in the mu4D5-treated group, compared with an overall occurrence rate of 54% in the control groups. Histologically, the enlarged Harderian glands showed residual normal-appearing acinar tissue adjacent to circumscribed areas of adenomatous growth (Fig. 5, C and D). In these latter areas, we observed an effacement of the normal glandular architecture with solid and papillary growth. There was increased cellular and nuclear size and pleomorphism, as well as mitotic activity among the epithelial cells (Fig. 5D). Invasion into adjacent

Fig. 3 Histological analysis of transgenic mammary tumors and associated pulmonary metastases. A, an H&E-stained section of mammary tumor tissue from the Fo5-transgenic mouse shows the classic histological features of an adenocarcinoma of the breast. B, immunohistochemistry with Herceptin reveals strong membranous staining for human (hu)HER2-transgenic protein on most tumor cells. C, photograph of an F2 female transgenic mouse (no. 196) of the Fo5 line illustrating a primary breast tumor just before tumor removal survival surgery. D, examination of the lung of mouse no. 196 at sacrifice reveals a metastatic tumor nodule from the same mammary tumor. Pulmonary metastases have been noted in our model in 23% of mice that have undergone survival tumor removal surgery. E, H&E-stained section of lung of representative animal no. 1807 shows metastatic mammary tumor nodules. F, these tumor nodules are strongly positive for huHER2 as determined by immunostaining with Herceptin. G, the primary mammary tumor of that same animal is a solid and papillary mammary adenocarcinoma as shown by H&E staining. H, immunohistochemistry with Herceptin shows strong and uniform expression of huHER2 in an adjacent section of mammary tumor.
stroma or distant metastases were not observed. The histological features of this lesion are most consistent with a classification as a Harderian gland adenoma, acinar type (29). As with mammary tumors, the Harderian gland neoplasms were determined by both immunohistochemistry and Taqman analysis to highly express huHER2 (Fig. 5, E and F).

Sequence Analysis of huHER2 Tumor Transcripts. Sequencing of amino acids 540–740 of the primary mammary tumor from the original Fo5-transgenic founder mouse revealed an in-frame 15-bp deletion (GACCTGGATGACAAG) in the huHER2 juxtamembranous region. This deletion resulted in the repositioning of two cysteines (634 and 641) to within two amino acids of each other, potentially affecting cysteine-mediated dimerization (Fig. 6A). Six independent reverse transcription reactions, followed by PCR and sequencing, revealed no additional transcript from this mammary tumor other than that of the deletion, consistent with a clonal tumor origin. In addition, complete sequencing of the entire Fo5 mammary tumor huHER2 RNA revealed no additional mutations in other regions of the huHER2 gene message.

Somatic in-frame mutations found in sequencing amino acids 540–740 in 48 mammary tumors from this model revealed a propensity for deletion (52%). More rare events such as insertions (4%), point mutations (18%), or a combination of deletion and point mutations (6%) were also seen. Sequence examination of transgenic mammary tumors was used to demonstrate the clonality of each primary tumor because >80% of independent mammary tumors carried a unique in-frame somatic mutation. Numerous transgenics were found to have developed several independent mammary tumors, each characterized by a unique mutation; hence, recurrence or local spreading of a tumor could be distinguished from the appearance of yet another primary tumor(s). Mouse no. 1790 from the mu4D5-treated group developed a primary breast tumor with a deletion of amino acids 643–652 (Fig. 6B). A second independent mammary tumor was next discovered with a deletion of amino acids 637–643. Pulmonary metastases from no. 1790 were also sequenced and found to derive from the original primary tumor (deletion amino acids 643–652). Similarly, sequencing of mammary tumors from mouse no. 1490 (not in the treatment study) revealed a deletion of a single amino acid 620 in the initial primary tumor, corresponding to the mutation in the pulmonary metastases, discovered at necropsy. A second independent mammary tumor was found to have a different, unique somatic mutation, an insertion of amino acids 632–635. Sequencing of the primary mammary tumor from mouse no. 1573, a member of the diluent control group, revealed a deletion of a single amino acid 620 in the initial primary tumor, corresponding to the mutation in the pulmonary metastases, discovered at necropsy. Hence, pulmonary metastases could be traced to the primary tumor of origin. In our model, we observed that pulmonary metastases tended to originate from the earliest appearing mammary tumor.

No sequence anomalies of the huHER2 transgene were detected in the juxtamembranous or transmembrane regions spanned by our primers in huHER2-overexpressing yet histologically normal-appearing mammary glands of young nontumor-bearing transgenic mice or in Harderian gland neoplasms. However, mutations were often found in normal-appearing mammary gland directly adjacent to mammary tumors.

Early Treatment of Female Fo5-Transgenic Mice With mu4D5. No significant weight variations were observed within or between treatment groups (data not shown). Determination of mu4D5 serum levels via HER2 ECD binding ELISA revealed that a level of at least 1 mg/ml circulating mu4D5 was
Fig. 5 Morphology and expression of human (hu)HER2 in Harderian glands of the Fo5-transgenic mouse line. No Harderian gland enlargement was observed in the mu4D5-treated group, compared with an overall incidence rate of 54% in the control groups. A, photograph of a transgenic female member of the mu4D5 treatment group, at study termination at 52 weeks of age, showing no effect of huHER2 overexpression in the Harderian gland, as compared with (B), a member of the diluent control treatment group who exhibits a Harderian gland tumor. C, H&E-stained section of an enlarged Harderian gland demonstrates adenomatous areas (upper two-third) adjacent to compressed residual acinar structures. D, the epithelial cells in adenomatous areas display nuclear enlargement and pleomorphism as well as mitotic figures (arrow). E, immunohistochemistry for huHER2 shows strong membranous staining of epithelial cells. The staining intensity is particularly strong in adenomatous areas. F, Taqman analysis for huHER2 transgene expression was performed on Harderian gland tissues from transgenic Fo5 female mice from this study. Analysis revealed a high level of huHER2 expression in enlarged Harderian glands in blue (from the control antibody and diluent control groups) and normal Harderian tissues in red (from the mu4D5 treatment group) when using the huHER2 probe set. Mammary gland tumor tissue was run as a positive control from mouse no. 1577 in our study (blue). Nontransgenic FVB mammary tissue was run as a negative control (red).
maintained in the serum of all mice in the mu4D5 treatment group throughout the study, with the exception of mouse no. 1528 (found dead at 45 weeks of age), whose mu4D5 level did not reach 1 mg/ml (data not shown). No serum mu4D5 was detected in the control treatment groups. In addition, there were no significant clinical chemistry or hematology findings noted in any treatment group.

Not surprisingly, 13 of 14 females (93%) in the control group treated with anti-gD antibody exhibited deleterious effects from huHER2 overexpression. Eight of the 14 mice (57%) developed mammary tumors at an average onset of 33 weeks, and 5 mice in the group were sacrificed because of morbidity from Harderian gland neoplasms at an average age of 45 weeks, with no mammary tumors noted (Table 1). The diluent/PBS control group was similarly afflicted. Although 1 mouse was found dead of unknown cause at 27 weeks with no mammary tumor, 8 of 11 mice (73%) developed mammary tumors. Seven had palpable tumors with an average onset of 36 weeks; one occult mammary tumor was found at study termination. Two other mice in this group had to be sacrificed because of morbidity from Harderian gland neoplasms, again with no evidence of mammary tumor development (Table 1). Overall incidence of lung metastases was 40% in these control groups. Aside from Harderian gland neoplasms, no primary tumors were found in tissues other than the mammary gland in the control groups throughout the 52-week study.

In contrast, early treatment with mu4D5 was found to significantly alter mammary tumor incidence and progression as compared with the control groups, suggesting a clear survival advantage. Only 4 of 14 mice (29%) in the mu4D5-treated group developed a mammary tumor during the study, 2 of which were identified by palpation, 1 at 23 weeks, and 1 at 44 weeks; both mice were found to have pulmonary metastases at necropsy. The other 2 mice with mammary tumors were not identified until the 52-week study termination necropsy, when each was found to harbor a very small mammary tumor (\(<1 \text{ mm}^3\); pulmonary metastases were not present. Overall incidence of pulmonary metastasis was 14% in this group. Two of the 14 mice in the mu4D5 treatment group were found dead of unknown causes at 30 and 45 weeks of age, respectively, and neither had mammary tumors.

The Kaplan-Meier disease-free survival plot for this study, illustrating weeks to mammary tumor onset, clearly reveals the significant survival advantage accorded the mu4D5-treated group of female transgenics as compared with the two control groups, whose tumor incidence curves, not surprisingly, overlap (log-rank test resulted in a \(P = 0.0267\), indicating significant difference between the mu4D5-treated group and the control groups; Fig. 7A). No tumors were evident in any other tissues in this group, and strikingly, Harderian gland neoplasms were entirely absent in all mice treated with mu4D5 (Table 1). In fact, the mu4D5-treated mice looked to be in exceptional condition.
with smooth, glossy coats and clear eyes up until study termination at 52 weeks (Fig. 5A).

Mammary tumor growth in both control groups followed the aforementioned characteristic pattern of the Fo5 line, as illustrated by mammary tumor growth curves from a representative anti-gD control antibody-treated mouse (no. 1807) and a representative diluent-treated mouse (no. 1573; Fig. 7B). The first mammary tumor from no. 1573 (tumor no. 1) grew continuously, reaching 1–1.5 cm$^3$ in a matter of weeks. After surgical removal, recurrence of the primary tumor (tumor no. 2) was noted. Another primary at an alternate location (tumor no. 3) was found at sacrifice, as were pulmonary metastases. As previously noted, one mammary tumor (in mouse no. 1790) was identified at 23 weeks in the mu4D5-treated group, revealing a unique 18-bp deletion, again in the juxtamembranous region of huHER2, confirming this was indeed a new clonal mammary tumor in this mouse (primary tumor no. 2; Fig. 6B). Mouse no. 1790 was the only mouse from the mu4D5 treatment group (aside from the 2 found dead of unknown causes) not to survive until the 52-week termination date. At necropsy, small pulmonary metastases were found in mouse no. 1790, with sequence corresponding to that of primary tumor #1. These metastases were noted by immunostaining to continue to express high levels of huHER2 (Fig. 7, E and F).

Mouse no. 1716, in the mu4D5-treated group, developed a primary mammary tumor that was discovered at 44 weeks by palpation; this mammary tumor remained stationary in size until study termination at 52 weeks (∼56 days later).

Table 1 A list of the individual Fo5 transgenic females that were participants in each treatment group, their mammary tumor status, Harderian neoplasm status, and if pulmonary metastases were noted upon study termination

<table>
<thead>
<tr>
<th>Mouse no.</th>
<th>Treatment</th>
<th>Mammary tumor onset/other</th>
<th>Pulmonary metastases</th>
</tr>
</thead>
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<tr>
<td>1528</td>
<td>mu4D5</td>
<td>Found dead at 45 wks.</td>
<td>No</td>
</tr>
<tr>
<td>1570</td>
<td>mu4D5</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>1613</td>
<td>mu4D5</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>1617</td>
<td>mu4D5</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>1628</td>
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<td>No</td>
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<td>1712</td>
<td>mu4D5</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>1716</td>
<td>mu4D5</td>
<td>44 weeks</td>
<td>Yes</td>
</tr>
<tr>
<td>1725</td>
<td>mu4D5</td>
<td>52 weeks</td>
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<tr>
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<td>mu4D5</td>
<td>Found dead at 30 wks.</td>
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</tr>
<tr>
<td>1731</td>
<td>mu4D5</td>
<td>52 weeks</td>
<td>No</td>
</tr>
<tr>
<td>1790</td>
<td>mu4D5</td>
<td>23 weeks</td>
<td>Yes</td>
</tr>
<tr>
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<td>mu4D5</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>1527</td>
<td>Control antibody</td>
<td>36 weeks</td>
<td>Yes</td>
</tr>
<tr>
<td>1569</td>
<td>Control antibody</td>
<td>38 weeks</td>
<td>Yes</td>
</tr>
<tr>
<td>1576</td>
<td>Control antibody</td>
<td>32 weeks</td>
<td>No</td>
</tr>
<tr>
<td>1611</td>
<td>Control antibody</td>
<td>31 weeks</td>
<td>Yes</td>
</tr>
<tr>
<td>1626</td>
<td>Control antibody</td>
<td>Hardenan-sac 44 wks.</td>
<td>No</td>
</tr>
<tr>
<td>1639</td>
<td>Control antibody</td>
<td>Hardenan-sac 44 wks.</td>
<td>No</td>
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<td>No</td>
</tr>
<tr>
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<td>Diluent control</td>
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<td>Diluent control</td>
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<td>1689</td>
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<td>Found dead at 27 wks.</td>
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</tr>
<tr>
<td>1717</td>
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<td>30 weeks</td>
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<tr>
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<td>Diluent control</td>
<td>34 weeks</td>
<td>Yes</td>
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<tr>
<td>1586</td>
<td>PBS control</td>
<td>48 weeks</td>
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</tr>
<tr>
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<td>PBS control</td>
<td>Hardenan-sac 42 wks.</td>
<td>No</td>
</tr>
<tr>
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<td>PBS control</td>
<td>Hardenan-sac 51 wks.</td>
<td>No</td>
</tr>
<tr>
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<td>41 weeks</td>
<td>No</td>
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<tr>
<td>1710</td>
<td>PBS control</td>
<td>30 weeks</td>
<td>Yes</td>
</tr>
<tr>
<td>1732</td>
<td>PBS control</td>
<td>Hardenan-sac 44 wks.</td>
<td>No</td>
</tr>
</tbody>
</table>

grow to the axilla, resulting in interference with mobility, thus requiring removal at a relatively small size (Fig. 7B). Sequence analysis of this primary tumor detected a 24-bp deletion in the juxtamembranous region of huHER2 (Fig. 6B). Histological examination revealed an adenocarcinoma with characteristic features (Fig. 7C); immunostaining with Herceptin confirmed the ability for antibody binding and showed the expected high level of huHER2 expression (Fig. 7D). After removal, a second rapidly growing mammary tumor > 1.5 cm$^3$ was found ∼2 weeks later. Sequence analysis of this second tumor revealed a unique 18-bp deletion, again in the juxtamembranous region of huHER2, confirming this was indeed a new clonal mammary tumor in this mouse (primary tumor no. 2; Fig. 6B). Mouse no. 1790 was the only mouse from the mu4D5 treatment group (aside from the 2 found dead of unknown causes) not to survive until the 52-week termination date. At necropsy, small pulmonary metastases were found in mouse no. 1790, with sequence corresponding to that of primary tumor #1. These metastases were noted by immunostaining to continue to express high levels of huHER2 (Fig. 7, E and F).

Mouse no. 1716, in the mu4D5-treated group, developed a primary mammary tumor that was discovered at 44 weeks by palpation; this mammary tumor remained stationary in size until study termination at 52 weeks (∼56 days later).

DISCUSSION

The formation of huHER2 overexpressing mammary tumors is a highly reproducible phenotype within the Fo5-transgenic line and this mouse model represents an opportunity to evaluate the in vivo efficacy of antibody-based anti-huHER2-targeted therapy. In this study, we chose the mu4D5 monoclonal antibody because it is the murine form of Herceptin, already in clinical use for patients with metastatic HER2-overexpressing breast cancer (15–17).

Our study demonstrates that early intervention with mu4D5, the murine form of Herceptin, led to a dramatic reduction in mammary tumor incidence in our transgenic mouse model. Obvious growth inhibition of those few tumors found in the mu4D5 treatment group provides additional evidence of the efficacy of mu4D5 in our study of early and prolonged treatment. In addition, Harderian gland neoplasms, commonly associated with overexpression of huHER2 in this transgenic model, were entirely absent in the presence of mu4D5 treatment, indicating down-regulation of huHER2 in vivo activity.

From this study, it appears that tumors that arose under mu4D5 treatment could continue to remain responsive to mu4D5, as evidenced by growth inhibition, for a period of time. However, in our study, very few tumors formed in the mu4D5-treated group, and only one tumor (no. 1790) appeared early enough (23 weeks) to demonstrate prolonged growth inhibition followed by a release from growth control. Interestingly, the second clonal mammary tumor that appeared in mouse no. 1790 exhibited no such mu4D5-mediated growth control. Additional studies are in progress to further analyze the characteristics of mammary tumors that arise during mu4D5 treatment. Understanding such mu4D5-mediated tumor growth inhibition, as well as factors that might contribute to subsequent loss of
Fig. 7 Characterization of transgenic treatment groups. A, the Kaplan-Meier disease-free survival plot for this study, illustrating mammary tumor onset in weeks, clearly reveals the significant survival advantage accorded the mu4D5-treated group (red) of female transgensics as compared with the two control groups [control antibody (Ab) in light blue and diluent/PBS in dark blue]. B, mammary tumor growth curves—the growth curves of representative mammary tumors from 3 mice are shown. C, H&E-stained section of the first mammary tumor of animal no. 1790 in the mu4D5 treatment group shows a characteristic adenocarcinoma. D, human HER2 immunohistochemistry of an adjacent section shows strong, uniform expression by the tumor cells. E, small pulmonary metastases were noted upon necropsy, with continued high expression of human HER2 detected by immunohistochemistry (F).
responsiveness, will be valuable in further optimizing the treatment of HER2-overexpressing cancers in the clinic.

Previous data from neu-transgenic models has demonstrated the presence of in-frame somatic mutations such as sequence deletions, confined to the juxtamembranous region of the neu ECD in mammary tumors (30, 31). These somatic mutations have been associated with the induction of mammary tumors via activation of intrinsic tyrosine kinase activity by the neu protein (32). The majority of these mutations affect the conserved cysteine residues in the region, implying that this region plays an important role in the catalytic activation of neu. These mutations could provide a second hit necessary for mammary tumor formation in addition to neu overexpression (30, 31). We therefore isolated total RNA from mammary tumors and tissues from our huHER2 Fo5 transgenics and performed RT-PCR followed by sequencing to look for similar mutations in the corresponding juxtamembranous region of huHER2, spanning amino acids 540–740. Like our colleagues, we also found sequence anomalies in the corresponding region of huHER2 in >80% of mammary tumors; deletion mutations were found as well as point mutations and insertions, all in-frame, resulting in aberrant huHER2 receptors. Less frequently, mutations were found located in the transmembrane domain.

If these huHER2 mutations truly function as a second hit in the transformation process in the female mammary gland, the observation of Harderian gland neoplasms without activating huHER2 mutations is puzzling, suggesting yet another second hit mechanism might be involved in this tissue. Sequencing of other second hit candidate genes such as p53 is in progress in normal and neoplastic Harderian glands and in normal-appearing mammary gland and mammary tumor tissue. In addition, sequencing of full huHER2 transcripts from Harderian gland neoplasms and mammary tumors found with no juxtamembranous mutations is in progress to confirm a lack of mutation(s) in other regions of the huHER2 message. Gene profiling analysis is also in progress to identify differences in gene expression in Fo5-transgenic tissues. Although the observation of somatic mutations in overexpressing mammary tumors is currently confined to transgenic mouse models of the HER2/ErbB family, it is clearly of interest to investigate the possible relevance to human breast cancer.

Cardiomyopathy has been shown to be a side effect of Herceptin treatment in 7% of women with HER2-overexpressing mammary tumors after treatment with first-line anthracycline therapy; when Herceptin is used concurrently with anthracycline treatment, the incidence of cardiac dysfunction reaches 28% (33). Although our transgenic mice from the Fo5 line were noted by Taqman analysis to express huHER2 in heart tissue (Fig. 3F), we found no gross histological or clinical evidence of cardiac or any other organ toxicity in the mu4D5-treated mice. In this study, mice in the mu4D5 treatment group received a relatively high weekly dose of mu4D5 (100 mg/kg) over an 8-month period, resulting in a serum titer of at least 1 mg/ml throughout the study duration, compared with the commonly used dose of 2 mg/kg Herceptin given i.v. every 3 weeks in the clinic (equivalent to ~10 mg/kg in mice). However, these mice were not treated with other therapeutic agents such as an anthracycline before or during this study.

It is clear from this study that early intervention with mu4D5 was of benefit in our transgenic model at high risk for developing huHER2-overexpressing breast cancer. With the obvious benefits of Herceptin treatment likely to expand to other patient populations, our transgenic mouse model will prove valuable in the elucidation of the molecular mechanisms and potential efficacies and toxicities involved in targeted therapeutic treatments, alone and in combination with other agents, of potential interest in the clinical treatment of huHER2-overexpressing breast cancer.

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REFERENCES


HER2-Targeted Therapy Reduces Incidence and Progression of Midlife Mammary Tumors in Female Murine Mammary Tumor Virus huHER2-Transgenic Mice

David Finkle, Zhi Ricky Quan, Vida Asghari, et al.


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