Spontaneous Feline Mammary Carcinoma Is a Model of HER2 Overexpressing Poor Prognosis Human Breast Cancer

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Abstract

Companion animal spontaneous tumors are suitable models for human cancer, primarily because both animal population and the tumors are genetically heterogeneous. Feline mammary carcinoma (FMC) is a highly aggressive, mainly hormone receptor–negative cancer, which has been proposed as a model for poor prognosis human breast cancer. We have identified and studied the feline orthologue of the HER2 gene, which is both an important prognostic marker and therapeutic target in human cancer. Feline HER2 (f-HER2) gene kinase domain is 92% similar to the human HER2 kinase. F-HER2–specific mRNA was found 3- to 18-fold increased in 3 of 3 FMC cell lines, in 1 of 4 mammary adenomas and 6 of 11 FMC samples using quantitative reverse transcription-PCR. Western blot showed that an anti-human HER2 antibody recognized a protein comigrating with the human p185HER2 in FMC cell lines. The same antibodies strongly stained 13 of 36 FMC archival samples. These data show that feline HER2 overexpression qualifies FMC as homologous to the subset of HER2 overexpressing, poor prognosis human breast carcinomas and as a suitable model to test innovative approaches to therapy of aggressive tumors. (Cancer Res 2005; 65(3): 907-12)

Introduction

Spontaneous tumors in cats and dogs, humans’ favored companions, are suitable models of human cancer (1). The relatively high incidence of some cancers, large body size, and shorter overall life span are advantageous factors. Furthermore, they share the same lifestyle as humans. Above all, companion animals and their tumors are genetically heterogeneous, as are randomly selected groups of cancer patients. Therefore, these models are interesting alternatives to rodents with transplantable tumors or to inbred animals with chemically induced tumors. However, to date, only fragmentary information is available on molecular alterations and biological behavior of companion animal tumors. This makes these tumors largely underused.

Tumors having potential relevance for human cancer biology include mammary carcinoma, osteosarcoma, melanoma, and leukemia/lymphoma. Among them, mammary tumors are frequent neoplasms in cats (≥10% all cases, 17% for females). Feline mammary carcinoma (FMC) shows age incidence, histopathology, and pattern of metastasis (2, 3) similar to human breast cancer. In particular, the lack of estrogen dependency in most tumors (4) suggests that FMC could be a suitable animal model for human hormonal unresponsive breast cancer. The isolated reports of overexpression and amplification of cyclin A (5) and p53 nuclear accumulation (6) might also indicate a similar tumorigenesis.

In many carcinomas, in both humans and animals, tyrosine kinase oncogenes are activated (7, 8). In human breast carcinoma, the HER2 gene is overexpressed in 15-25% of cases (9). The HER2 (also known as erbB-2, neu) gene encodes a M, 185,000 transmembrane glycoprotein that is a member of the epidermal growth factor receptor (erbB) family of receptor tyrosine kinases. In most breast carcinomas, HER2 overexpression/amplification is known to confer poor prognosis (9-11) and may also predict insufficient response to hormonal therapy and standard chemotherapy regime (12). Therefore, HER2 is widely considered a key oncogene involved in human breast carcinoma onset and progression and its overexpression is still the most important marker of prognosis. The success of HER2 receptor-targeted therapy with monoclonal antibodies (13, 14) and the perspective of anti-HER2 vaccines in preventing primary breast cancer or relapse (15), make this oncogene also the best target of innovative therapeutic approaches in human breast cancer.

Here we propose FMC as a model of human breast cancer, based on frequent overexpression of the feline orthologue of HER2. We have previously reported the study of the RON tyrosine kinase receptor in cat mammary carcinoma (16), which already suggested this similarity. Here we describe the identification of the feline HER2 (f-HER2) gene; by quantitative reverse transcription-PCR (RT-PCR) and immunohistochemistry, we showed that f-HER2 expression is barely detectable in the normal cat mammary gland, is increased in mammary benign tumors, and is elevated in a high percentage of carcinoma samples.

Materials and Methods

Cell Lines. FRM and FYC (17, 18) were grown in RPMI medium and K248P (19) cells in DMEM medium plus 10 µg/mL insulin. In all cases, media were supplemented with 10% fetal calf serum, 100 µg/mL penicillin, 100 µg/mL streptomycin, and 1.5 mg/mL fungizone. SKBR3, Fc3Tg, Fc2Lu, and AK-D cells were obtained from American Type Culture Collection (Rockville, MD).
DNA and RNA Extraction and Sequencing; End Point and Quantitative RT-PCR. Genomic DNA and RNA were extracted and sequenced as described (16). The feline HER2 DNA sequence has been submitted to Genbank (accession no. AY685128). To determine the amount of feline HER2 transcript, quantitative RT-PCR was done using the ABI Prism 7700 Sequence Detection System (Applied Biosystem, Foster City, CA) and specific probe. As reference, the feline β-glucuronidase mRNA was measured using Syber Green. Probe and primers used are available from the authors.

Western Blot and Immunohistochemistry. For Western blot analysis, 200 μg of total protein from all samples but SKBR3 cells (50 μg) were loaded into gels. Immunohistochemical evaluation of HER2 protein was carried out on 4-μm sections of formalin-fixed, paraffin-embedded tissues, as previously described (16). Cultured cell pellets were similarly fixed, embedded, and sectioned. Pathologic classification was in accordance with WHO criteria (20). In all experiments, feline HER2 protein was labeled with anti-HER2/neu (A0485, Dako Cytomation, Glostrup, Denmark). Immunohistochemistry required antigen retrieval in citrate buffer 10 mmol/L (pH 6.0) for 40 minutes at 94°C. Control experiments using unrelated antibody, secondary antibody alone and horseradish peroxidase-conjugated Protein-A alone were done.

Results

Identification of Feline HER2 Gene. To identify cat HER2 sequences, we compared the coding sequences of the human and canine HER2 cDNAs. Strong similarities have been described among tyrosine kinase receptors of the same family in different species (21). Human and canine HER2 cDNAs show evident similarity, in particular in the kinase domain (Supplementary Fig. 1). As the human HER2 kinase domain shows also similarities with that of the ERBB family, to amplify feline HER2 DNA, we designed primers in regions that differ as much as possible from the corresponding of the other human ERBB family receptor genes.

From genomic DNA extracted from cat lung tissues we amplified and directly sequenced PCR products using primers designed on human HER2 gene exons 17 to 23. The feline DNA sequence obtained is shown in Supplementary Fig. 2, where it is aligned with the human sequence. Strong similarity (92%) was found between human 17 and 23 exon sequence and the amplified feline DNA.

Identification of Feline HER2 mRNA. To analyze mRNA encoded by the f-HER2 gene, primers were designed on the feline DNA putative exons, identified on the basis of similarity between feline DNA (Supplementary Fig. 2) and human sequence encompassing exon 17, encoding HER2 receptor transmembrane domain, and exon 23, encoding a part of the receptor kinase domain. CDNA was obtained from retro-transcription of total RNA isolated from cat mammary gland. The obtained PCR product was sequenced and f-HER2 intron-exon boundaries were defined (Supplementary Figs. 1 and 2). As previously mentioned, the amplified f-HER2 gene coding sequence showed strong similarity to human exon 17 to 23, whereas intron sequences differed. The f-HER2 sequence coding a feline putative receptor intralocular domain was 88% similar to the corresponding domain of mouse neu and 94% to that of dog HER2 (Supplementary Fig. 1).

Expression of Feline HER2 in Mammary Carcinoma Cell Lines. To measure f-HER2 expression, cDNA was amplified from retro-transcribed mRNA extracted from three feline cell lines propagated from normal tissues (AK-D, Fc3Tg, and Fc2Lu) and three FMC (K248P, fyc, and frm) cell lines (17–19, 22). The AK-D and Fc2Lu were propagated from feline lung samples and described as epithelial cell lines; the Fc3Tg were from cat embryonal tongue and described as a fibroblast-like cell line. The K248P cell line was established from a primary FMC and shown to be tumorigenic and metastatic in a xenograft model; the fyc cells were propagated from a primary FMC; the frm cells were from the pleural effusion of a FMC. We used quantitative RT-PCR with Taqman assay to measure the amount of f-HER2 mRNAs from cell lines and to compare it to mRNA extracted from a pool made of three normal mammary glands, harvested from nonovariectomized cats. As shown in Fig. 1A, carcinoma cell lines showed a 3- to 18-fold increase of f-HER2 specific mRNA.

To assess if f-HER2 increased expression in mammary carcinoma cell lines was associated to gene amplification, genomic f-HER2 was measured with quantitative PCR. Amplification was not found in any of the cell lines (data not shown). To assess if the f-HER2 mRNA gives rise to a protein product, we extracted total proteins from the three FMC cell lines and cell lines propagated from cat normal tissues (Fig. 1B). Proteins were separated under reducing conditions in SDS-PAGE gels. As feline and human HER2 mRNAs showed strong similarity in the intracellular domain (Supplementary Fig. 1), in Western blot analysis we used purified polyclonal antibodies raised against the human intracellular HER2 domain. The antibody labeled a band of the approximate Mr of 185 kDa in all but one (Fc3Tg) cell line, which comigrates with the p185 HER2 human protein overexpressed by the SKBR3 breast cancer cell line (Fig. 1B). It is noteworthy that only cells showing fibroblast-like phenotype cells did not express the p185 protein.

As anti-human HER2 antibody can label a feline comigrating protein, we used the same antibody to study the localization of the f-HER2 protein in the mammary carcinoma cell lines by immunohistochemistry. This antibody stained the HER2 protein in the human breast cancer cell line SKBR3, where the overexpressed protein is detectable in both the cytoplasm and cell membrane (Fig. 2). The antibody stained also the three FMC cell lines with different intensities, the highest being K248P cells that express the highest HER2 mRNA level (Fig. 2). In the latter cells, the labeling of membrane associated HER2 protein was more evident.

Expression of f-HER2 in Cat Mammary Tumors. To evaluate the f-HER2 expression in a panel of mammary carcinoma samples, specific transcripts were measured by quantitative RT-PCR. We purified cytoplasmic mRNA from four benign and 11 malignant mammary tumors harvested from nonovariectomized queens; malignant tumors were six solid and five tubulo-papillary adenocarcinomas.

Tissues were harvested and snap frozen at surgery. Figure 3 shows that f-HER2 transcripts were more abundant in 1 of 4 adenomas and 6 of 11 (55%) carcinomas than in normal mammary gland tissues.

As a limited amount of cat mammary carcinoma tissues was available for either RNA or protein extraction, expression of the f-HER2 protein in a large series (40 cases) of tumors was surveyed using archival samples and immunohistochemistry. This series included all the samples analyzed with quantitative RT-PCR. The study was carried out using the anti-human HER2 antibody selected for its ability to recognize feline HER2 protein, as described in the previous paragraph.

We examined also five normal mammary gland samples and a hyperplastic mammary gland, which did not show labeling (Fig. 2 and data not shown). Immunohistochemical f-HER2 protein staining of samples already analyzed with quantitative RT-PCR showed that only tumors displaying a 3-fold increase of f-HER2 mRNA express detectable amount of protein (data not shown). Again, only 1 of 4 adenomas was positive (Fig. 2). Five of 10 solid carcinomas and 10 of 26 tubulo-papillary carcinomas were
strongly positive (Fig. 2). Collectively, 39% of carcinomas showed a strong staining with the anti-human HER2 antibody. In most cases, staining was homogeneously distributed along the section and showed comparable intensities, being in some cases more evident the membrane-associated staining (Fig. 2). Although staining intensity was not semiquantitatively scored, we noticed that the labeling was more evident in tumors already classified as strong expressors using quantitative RT-PCR.

Discussion

Breast cancer is the most frequently diagnosed cancer in women and the second most frequent cause of cancer death. Amplification of the HER2 gene, resulting in overexpression of the receptor, is found in 15% to 25% of human breast cancers, a frequency of genetic alteration that is second only to p53 mutations (9). HER2 gene overexpression is an important prognostic marker and an unique target for molecular-directed therapy. Here we show that FMC show f-HER2 overexpression with a high frequency. As 90% of FMCs are malignant and show a 1-year survival rate of 50% (23), FMC qualifies as a suitable model of HER2 overexpressing, aggressive, and poor prognosis human breast cancer.

We studied three FMC cell lines and identified feline HER2 DNA and protein. We measured mRNA expression in 16 FMC samples and found increased expression in 55% of them, including one mammary adenoma. Among these samples, those showing at least a fold increase of f-HER2 mRNA scored positive in immunohistochemistry, carried out using a cross-reactive anti-human HER2 antibody. As already shown in human cancer (24), a good correlation was found between mRNA expression and protein expression. However, tissue heterogeneity in cat and human samples lowers the estimation of the amount. Immunohistochemistry carried out on a larger FMC series showed that ~40% were strongly HER2 positive. Negative scored samples could not be easily estimated due to weak positivity, likely because of the presumed lower affinity of the antibody raised towards the human protein. In addition, archival samples of companion animal tumors are frequently fixed and embedded using different protocols and it is known that immunohistochemistry sensitivity highly depends on fixative conditions. In conclusion, positive cases were certainly underestimated rather than overestimated.

In >90% of HER2 overexpressing human breast cancers the gene is amplified. In FMC cell lines, increased messenger RNA is not associated to gene amplification, but we show that increased mRNA expression results in strong protein expression. This means that other mechanisms than gene amplification might regulate HER2 expression in FMC, as also shown in dogs (25).

Dog mammary carcinomas are highly heterogeneous, more than FMC, as far as clinical outcome and molecular profiling are concerned (26). Only approximately half of dog mammary tumors are malignant and a small percentage show canine HER2 increased expression (25, 27). A correlation was reported between HER2 overexpression mammary carcinoma and unfavorable prognosis (25). Although case numbers were usually small, these data suggest that HER2 gene activation accompanies dog mammary cell transformation, as in humans.

FMC is highly aggressive, growing rapidly, and metastasizing to regional lymph nodes and to lung (3), as the most malignant tumors in humans. In FMC conventional chemotherapy is minimally effective. FMC proliferation indexes (28) and grading (23) have been correlated to aggressiveness. The majority of FMC are classified as either tubulo-papillary or solid adenocarcinomas (20), like the human ones. Most FMC express low level of both estrogen (4) and progesterone (29) receptors. Most FMC respond only partially to ovariectomy or anti-hormone therapy (30), as the more aggressive human breast carcinomas. In human breast cancers, an inverse association has been described between HER2 amplification/overexpression and the presence of receptors for the steroid hormones estrogen and progesterone in both clinical correlative studies (31) and experimental models (32). Consistent with this negative association, HER2 amplification/overexpression has been associated with the failure of endocrine treatment, such as tamoxifen therapy, in retrospective studies. Clinical data have also suggested that HER2 amplification/overexpression might be associated with tamoxifen resistance in patients with estrogen
Figure 2. F-HER2 protein expression and localization in FMC cell line and tissues. Immunohistochemical detection of HER2 protein in formalin-fixed, paraffin-embedded cell pellets (A), nonmalignant tissues (B), and FMC (C-D) using anti-HER2 polyclonal antibody. A, K248P FMC (left) and SKBR3 human breast cancer cells (right), ×400. B, nonmalignant feline breast tissues: normal mammary gland from nonovariectomized cat (left) and a positive cat mammary fibroadenoma (right), ×400. C, tubulo-papillary FMCs: a negative sample (left) and a positive sample (right) at lower (×200, middle) and at higher magnification (×400, right). D, solid FMCs: two positive solid carcinomas; middle, a lower magnification (×200) of the sample on the right at higher magnification (×400).
receptor–positive breast cancer (33). Altogether, these data suggest that FMC represents a model of HER2 overexpressing and hormone-independent human breast carcinomas.

Other phenotypic characteristics suggest an analogy between FMC and aggressive human breast carcinoma. More than 70% of FMCs show strong reduction or absence of E-cadherin that is expressed in the cat normal gland (34). Expression of vascular endothelial growth factor, which in human breast carcinoma was correlated to poor prognosis, has been reported in all FMC and correlated with unfavorable prognosis (35). Conflicting reports failed to depict p53 status of FMC: p53 protein expression was found increased in 35% to 45% of FMCs (6, 36), whereas mutations were detected in only one sample out of a small series when exons 4 to 8 were sequenced (37). However, it is worth noting that prevalence of p53 mutations in human breast cancers was 40% to 70% only when all the exons were analyzed (38). RAS mutations were not found in FMC (39), consistently with the absence/low prevalence of RAS mutation in human breast cancer (40).

Spontaneous tumors in companion animals have been repeatedly proposed as appropriate and valid model tumor systems, but critical genetic and molecular information still lack. Cat (41) and dog genome (42) sequencing change the perspective. In particular, studying new chemotherapeutic regimens, gene therapy, immunotherapy, and biological modifications in companion animals with naturally occurring cancer have many advantages over more conventional models. In this work, we show that FMC presents the additional advantage of being similar to a well-defined subset of breast cancers, those showing HER2 overexpression, absence of hormone receptors, aggressiveness, and poor prognosis that require innovative therapeutic approaches.

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