

# C35 (*C17orf37*) is a novel tumor biomarker abundantly expressed in breast cancer

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## Abstract

Identification of shared tumor-specific targets is useful in developing broadly applicable therapies. In a study designed to identify genes up-regulated in breast cancer, a cDNA clone corresponding to a novel gene **C35** (*C17orf37*) was selected by representational difference analysis of tumor and normal human mammary cell lines. Abundant expression of C35 transcript in tumors was confirmed by Northern blot and real-time PCR. The **C35** gene is located on chromosome 17q12, 505 nucleotides from the 3' end of the *ERBB2* oncogene, the antigenic target for trastuzumab (Herceptin<sup>TM</sup>) therapy. The chromosomal arrangement of the genes encoding C35 and *ERBB2* is tail to tail. An open reading frame encodes a 12-kDa protein of unknown function. Immunohistochemical analysis detected robust and frequent expression of C35 protein, including 32% of grade 1 and 66% of grades 2 and 3 infiltrating ductal carcinomas of the breast (in contrast to 20% overexpressing *HER-2/neu*), 38% of infiltrating lobular carcinoma (typically *HER-2/neu* negative), as well as tumors arising in other tissues. C35 was not detected in 38 different normal human tissues, except Leydig cells in the testes and trace levels in a small percentage of normal breast tissue samples. The distinct and favorable expression profile of C35 spanning early through late stages of disease, including high frequency of overexpression in various breast carcinoma, abundant expression in distant metastases, and either absence or

low level expression in normal human tissues, warrants further investigation of the relevance of C35 as a biomarker and/or a target for development of broadly applicable cancer-specific therapies. [Mol Cancer Ther 2006;5(11):2919–30]

## Introduction

Breast cancer is the most common cancer and is the second leading cause of cancer-related deaths in women living in the United States. More than 40,000 women in the United States will die of breast cancer this year, amounting to ~15% of all cancer-related deaths (1). Identification of cancer-specific biomarkers has enormous potential to enhance detection, treatment, and prognosis of breast cancer. In addition, understanding the role of such biomarkers in the process of transformation could reveal opportunities to target cancer-specific proteins therapeutically and increase treatment options for breast cancer.

The advantages of exclusively targeting malignant cells are clear; yet, few targeted therapies have been successful for the treatment of solid tumors. One cancer-targeted therapy (Herceptin<sup>TM</sup>) has been a notable and encouraging advance in targeted treatment of solid tumors that overexpress the *HER-2/neu* receptor. However, the numbers of patients who are eligible and responsive to Herceptin are few, highlighting the need for new, more broadly applicable targets.

Herein, we describe identification of a novel biomarker termed C35 and initial analysis of its gene structure. Furthermore, through generation of anti-C35 antibodies, extensive studies of protein expression in a panel of human breast carcinoma and normal human tissues show specific and abundant expression in early-stage and late-stage disease. Expression of C35 is compared with expression of *HER-2/neu*.

## Materials and Methods

### Cell Lines and Culture

Human mammary carcinoma cell lines BT-20, BT-474, MCF7, MDA-MB-231, SK-BR-3, T-47D (supplied by the American Type Culture Collection, Manassas, VA) and PCI-13 (2), a head and neck carcinoma provided by Theresa Whiteside (University of Pittsburgh Cancer Institute), were grown in RPMI 1640 (Bio Whittaker, Walkersville, MD) with 10% fetal bovine serum (Biofluids, Rockville, MD). An immortalized line derived from normal breast epithelium (H16N2), two metastatic breast carcinoma lines (21MT-1 and 21MT-2), and two primary tumor lines (21NT and 21PT) were provided by Dr. Vimla Band (Tufts University School of Medicine). H16N2 and the four 21-T series tumors were derived from the same patient with infiltrating and intraductal carcinoma and grown in DFCI (3, 4).

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Colau 467p colon adenocarcinoma cells, grown in DMEM/F12, 10% fetal bovine serum, 100  $\mu\text{mol/L}$  nonessential amino acids, were provided by Drs. Maha Ayyoub and Danila Valmori (Ludwig Institute for Cancer Research, New York). MEL1700 melanoma cell line, provided by Dr. Steven Rosenberg (National Cancer Institute, NIH), were grown in RPMI, 20% fetal bovine serum.

#### RNA Extraction and Northern Blot Analysis

Cells were harvested at  $\sim 80\%$  confluency; RNA was extracted as per manufacturer's protocol (Qiagen RNeasy kit). Snap-frozen human tissues, provided by the Cooperative Human Tissue Network (funded by the National Cancer Institute), were homogenized directly in QG lysis buffer. For Northern blots, mRNA was isolated using oligo-dT<sub>25</sub> magnetic beads (DynaL, Lake Success, NY) and electrophoresed (30  $\mu\text{g/well}$ ) in 0.8% agarose, 3% formaldehyde and blotted onto Genescreen Plus (Perkin Elmer Life and Analytical Sciences, Inc., Wellesley, MA) in  $10\times$  SSC. Multiple-Tissue Northern blot was purchased from Clontech Laboratories (Palo Alto, CA). cDNA probes were random-primed to incorporate <sup>32</sup>P (Prime-It, Stratagene, La Jolla, CA) at  $10^6$  cpm/mL in Quickhyb solution (Stratagene), as per manufacturer's protocol. All blots were normalized for loading by probing with housekeeping gene *GAPDH* or  *$\beta$ -actin*.

#### Subtractive Hybridization

PCR Select cDNA Subtraction kit (Clontech Laboratories), based on representational difference analysis as first described by Lisitsyn et al. (5), was employed to generate cDNAs enriched for genes overexpressed in breast tumors compared with normal breast cell line H16N2. Products were cloned into pT7Blue3 (Novagen, Madison, WI) to generate a subtracted library. Clonal inserts were PCR amplified from overnight Luria-Bertani/ampicillin (100  $\mu\text{g/mL}$ ) cultures, and PCR products were spotted on Genescreen Plus using BioDot manifold (Bio-Rad Laboratories, Hercules, CA). Duplicate dot blots were probed with random-primed tumor or normal cDNA, or, alternatively, the PCR products of the forward (tumor minus normal) and reverse (normal minus tumor) subtractive hybridizations. Clones that seemed to be overexpressed in the tumor cDNA and forward subtraction were analyzed by Northern blot to confirm differential gene expression.

#### Sequencing

Automated sequencing was done with T3 and T7 primers by LoneStar Labs, Inc. (Houston, TX).

#### Isolation of Full-length Gene

To isolate the coding region of C35, oligo-dT primed double-stranded cDNA was generated from SK-BR-3 using SMART cDNA Synthesis (Clontech Laboratories). Primers were synthesized (C35 sense, 5'-GCGATGACGGGGGA-GCC and C35 antisense, 5'-CCACGGAATCTTCTATTCT-TTCT; Fisher Oligos, The Woodlands, TX) based on the deduced open reading frame. The start of transcription was identified using 5' rapid amplification of cDNA ends (Generacer, Invitrogen, Carlsbad, CA) of RNA isolated from normal human placental tissue and was confirmed using RNA from human breast and liver tissue.

#### Real-time PCR

Total RNA was isolated using an RNeasy miniprep kit (Qiagen, Valencia, CA) and treated with RNase-free DNase I at 37°C for 30 minutes (Ambion, Austin, TX). First-strand cDNA synthesis was done using Superscript II (Invitrogen, Carlsbad, CA). PCR primers were biased toward small size (75–125 nucleotides), close proximity to the 3' end of the transcript, and 60°C annealing temperature. Primer sequences are as follows; human  $\beta$ -actin sense, CCTTTTGTCCCCCAACTT; human  $\beta$ -actin antisense, TT-CAACTGGTCTCAAGTCAGTGT; human C35 sense, AAAGATCTCATTGAGGCCATCC; human C35 antisense, GAGACCAAGGTTTGGACCCC; human ERBB2 sense, AAGAGTCCCAACCATGTCAA; human ERBB2 antisense, AAAAGTCATCAGCTCCACA. Before use in a quantitative assay, all primer pairs were qualified for their ability to produce a single PCR product and the absence of primer-dimer on agarose gel using the same amplification conditions used for real-time PCR. A custom  $10\times$  QPCR Master Mix buffer included Taq DNA polymerase, deoxynucleotide triphosphates, and  $\text{MgCl}_2$  (Abgene, Surrey, United Kingdom), 0.2  $\mu\text{mol/L}$  primers. ROX (250 nmol/L) was used as an internal reference and 1:40,000 dilution of SYBR Green (Molecular Probes®, Invitrogen). Real-time PCR was done in triplicate on a Prism 7900 (Applied Biosystems, Foster City, CA). Specificity of the amplification was monitored by dissociation curves. The dissociation of SYBR Green-labeled cDNA was done after completion of PCR by heating the products for 15 seconds at 95°C and 20 seconds at 60°C and increasing the temperature slowly up to 95°C over a 20-minute interval. The dynamic range and efficiency of all primer pairs were tested using serial RNA dilution to generate a relative standard curve. RQ-PCR data were analyzed using a comparative relative quantification method (6). Change in gene expression was normalized with the  $C_T$  value of  $\beta$ -actin rRNA used as endogenous control. When measuring genomic amplification status, intra-sample normalization was achieved by assuming that the  $\beta$ -actin locus remains unamplified between samples and during tumorigenesis.

#### Luciferase Assays

Reporter gene plasmid (C35PR-Luc) was constructed including the 351-bp promoter region of C35, surrounding the start of transcription at positions  $-306$  through  $+45$ . Cells were seeded at  $5 \times 10^5$  per well in a six-well plate the day before transfection; 1  $\mu\text{g}$  of reporter gene plasmid (C35PR-Luc) and 20 ng of control plasmid (pRL-SV40) were premixed with LipofectAMINE Plus (Invitrogen). Twenty-four hours after transfection, the cells were harvested, and a Dual-Luciferase reporter assay system (Promega, Madison, WI) was used for sequential measurement of firefly and *Renilla* luciferase activities with specific substrates. Quantification of luciferase activities and calculation of relative ratios were carried out using a luminometer (MicroLumiXS, Harta Instruments, Gaithersburg, MD); at least three independent transfections were done in triplicate.

### Recombinant Protein

The coding region for human C35 was cloned into pet28 vector (Novagen), including a 6× his tag, and transformed into the BL21(DE) *Escherichia coli* strain. Induced cultures were lysed with BugBusterHT Protein Extraction Reagent and purified using NiNTA binding resin (Novagen). Purified recombinant human C35 protein was eluted in 50 mmol/L NaH<sub>2</sub>PO<sub>4</sub>, 300 mmol/L NaCl, 250 mmol/L imidazole (pH 8); imidazole was removed using a PD-10 desalting column. The his tag can be cleaved from the recombinant protein via thrombin digestion.

### Polyclonal Anti-C35 Antibodies

Rabbits were immunized with recombinant his-tagged C35 protein and purified by affinity chromatography against thrombin-cleaved C35 protein. Two polyclonal antibodies (B78-2 and B709129) were independently generated (Bethyl Laboratories, Montgomery, TX) and confirmed. Both antibodies showed the same specificity on rC35 and a panel of normal and malignant tissues using several assays, including ELISA, Western blot, and immunohistochemistry.

### Western Blot Analysis

rC35 (100 ng) and/or cell lysates (100,000 cell equivalents per lane) were electrophoresed on 18% Criterion gels (Bio-Rad Laboratories) in 1× TGS (25 mmol/L Tris, 192 mmol/L glycine, 0.1% SDS). Gels were transferred to Immobilon P<sup>5Q</sup> (Millipore, Bedford, MA) in 1× TG, 20% methanol in a Bio-Rad Criterion transfer chamber. Membranes were blocked overnight at 4°C in TNT [25 mmol/L Tris-HCl (pH 8), 150 mmol/L NaCl, Tween 20] + 5% Carnation nonfat dried milk. Primary antibody horseradish rabbit antiserum (1 µg/mL) and secondary antibody horseradish peroxidase-conjugated goat anti-rabbit IgG (Zymed®, Invitrogen; 1:20,000) were incubated for 1 hour at room temperature. Signal was detected with ECL-Plus (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) and exposed to X-OMAT AR film.

### Immunohistochemistry

Staining of formalin-fixed, paraffin-embedded breast carcinoma and normal tissue sections was done in part by QualTek Molecular Labs (Santa Barbara, CA). Ten lobular and 50 invasive ductal (grades 1, 2, and 3) carcinomas were selected from the QualTek tissue bank. Whenever possible, specimens were selected to include areas of ductal carcinoma *in situ* (DCIS) and/or normal-appearing adjacent tissue. Samples ranged in degree of lymph node involvement and in tumor size from 0.5 to 3.5 cm. Patient age range was 46 to 77 years, with a median of 57 years and mean age of 59 years. Specimens were qualified based on grade, pathologist's assessment of morphologic criteria, and positive immunoreactivity to a positive control antibody specific for Ki-67. Infiltrating ductal breast carcinomas (IDC) were graded according to a modified Scarff-Bloom-Richardson system, according to histologic differentiation (tubule formation), the degree of nuclear atypia, and mitotic count (based on Ki-67 staining). Normal breast samples were derived from both cancer and non-cancer biopsies. Samples of other normal human tissues

(*n* = 3–5) were selected from non-cancer biopsies based on pathologist's morphologic assessment and reactivity to a positive control antibody specific for vimentin, an antibody reactive to intermediate filaments.

AccuMax Array A202(III) (ISU ABXIS Co., Ltd., Seoul, Korea) and Asterand TMA 195 Breast Cancer (Asterand, Detroit, MI) tissue arrays were stained with B78-2 rabbit polyclonal, or rabbit IgG control following antigen retrieval with 0.05% saponin, 0.05% Tween 20 in PBS. AccuMax Array consisted of 30 IDCs, including 4 samples with matched normal tissue, 4 infiltrating lobular carcinoma (ILC), 8 cases of papillary carcinoma, and 3 phyllodes sarcoma. Samples contained on the AccuMax Array were derived from Korean patients, including one male, ranging in age from 36 to 78 years (median = 46 years, mean = 51 years). Asterand TMA array included three hundred sixty 0.6-mm cores; each of 120 samples were spotted in triplicate. Samples were excised in the United States between 2001 and 2004. Female patients ranged in age from 26 to 85 years, with a mean and median age of 57 years. Adjacent normal breast from nine diseased patients were included with 71 infiltrating ductal, 22 infiltrating lobular, 2 mucinous, and 6 medullary carcinomas. Stage information was also provided on the IDC of the Asterand and ISU ABXIS arrays.

Four-micrometer sections were deparaffinized through changes of xylene and rehydrated by a series of graded alcohol to distilled water. Pretreatment included steam heat-induced epitope recovery in citrate buffer (pH 6) with proteinase K digestion. Following avidin-biotin blocking procedure, sections were incubated with primary antibody against rabbit anti-C35 antibody B709129, or rabbit IgG (1 µg/mL) for 1 hour at room temperature. For competition experiments, samples were preincubated with a mixture of anti-C35 antibody and an excess of recombinant protein (C35 or β-galactosidase). ERBB2 staining was done on formalin-fixed, paraffin-embedded sections with 20 minutes of steam heat epitope-induced retrieval in pH 6 citrate buffer followed by incubation with antibody to c-erb-B2 oncoprotein (1:750; Novocastra, New Castle upon Tyne, United Kingdom) at room temperature. Herceptest<sup>TM</sup> (DAKO North America, Inc., Carpinteria, CA) was used to stain arrays, with protocol as described by manufacturer. An avidin-biotin-based tissue staining system was used for detection, and horseradish peroxidase was used as a reporter enzyme with 3,3'-diaminobenzidine as chromagen. Counterstaining with hematoxylin to assess cell and tissue morphology was followed by dehydration through graded alcohol series and xylene rinses.

Formalin-fixed, paraffin-embedded cell pellets from H16N2 and 21MT1 were used repeatedly as controls for all immunohistochemistry experiments. Sections were reviewed by light microscopy and confirmed independently by two qualified pathologists. Samples were evaluated for C35 reactivity based on an intensity scale of 1 to 4. Samples were considered positive with a 2+ score or higher and with cytoplasmic staining in >50% of tumor cells. For ERBB2 reactivity, a positive score of 2+

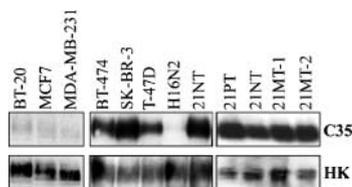
was defined by HercepTest (Dako North America Inc., Carpinteria, CA) guidelines, including complete membrane staining in >10% of cells. Cytoplasmic staining with anti-erb-B2 antibody is not considered positive using these guidelines.

## Results

### Differential Gene Expression Analysis

A subtractive hybridization was done to identify genes that were overexpressed in breast cancer relative to nontumorigenic breast epithelial cells. Using subtractive hybridization (5) coupled with suppression PCR (7), a subtracted library was generated that was enriched for cDNA fragments overexpressed in the breast cancer cells. Northern blot analysis confirmed differential expression and size of the RNA transcripts in several mammary cell lines and tissue specimens from various tumors and normal tissues. A preliminary screen identified clones representing at least 11 independent genes that were overexpressed in breast tumor cell lines. Some of these sequences were homologous to genes previously reported to be overexpressed in cancer, including human hypoxia inducible factor (accession no. NM\_001530), a putative microvascular endothelial cell differentiation gene (accession no. NM\_012328), an anti-trypsin protease (accession no. NM\_000295), and a serine protease kallikrein 6 (accession no. NM\_002774; ref. 8). Several novel genes were also identified, including C35, which were overexpressed at levels > 10-fold in the breast tumors.

Clone C35 was deemed to be of particular interest, as transcript levels were significantly increased in tumors when compared with normal tissues. Based on densitometric analysis of Northern blots, it was determined that clone C35 mRNA is expressed in 7 of 10 human breast carcinoma cell lines at levels 10- to 70-fold higher than the normal immortalized breast epithelial cell line H16N2 (Fig. 1). Greater than 10-fold expression was also found in RNA extracted from human breast (12 of 17) and bladder (3 of 14) cancer, whereas other carcinomas, such as colon (2 of 15) and prostate (8 of 11), were only slightly positive by Northern blots. In all C35<sup>+</sup> samples, a full-length probe hybridized to a single transcript of ~800 nucleotides, suggesting absence of splice variants. The faint signals seen in cell lines H16N2, BT-20, MCF7, and MDA-MB-231 was



**Figure 1.** Northern blots showing mRNA expression in mammary carcinoma cell lines, compared with H16N2, an immortalized cell line derived from normal breast epithelial tissue. The <sup>32</sup>P-labeled probe consisted of the open reading frame of C35 transcript (top). C35 expression in each cell line is normalized to housekeeping (HK) gene  $\beta$ -actin (bottom left and middle) and/or GAPDH (bottom right).

considered to represent the basal level of RNA expression, and overexpression was quantitated relative to this low level. Overexpression of C35 transcript was observed among several independently derived human mammary carcinoma cell lines, including SK-BR-3, T-47D, and BT-474. In addition, C35 expression is shared among lines derived from both primary (21NT and 21PT) and metastatic (21MT-1 and 21MT-2) lesions of a single patient.

### Gene Expression and Analysis

The full-length C35 transcript (Genbank accession no. AY508814) is 776 nucleotides and includes an open reading frame encoding a putative 115-amino-acid protein (Fig. 2). The C35 gene maps to human chromosome 17q12 (NT\_010755), position 35,138,935 to 35,140,314 (9), and is comprised of four exons (Fig. 3). C35 is located 505 nucleotides away from the oncogene *ERBB2* on the centromeric side in a tail-to-tail arrangement and 7402 nucleotides from the *Growth Factor Receptor-Bound Protein 7* gene (*GRB7*; refs. 10, 11) on the telomeric side, in a hotspot for gene amplification on human chromosome 17.

We have done sequence analysis of the C35 gene in primary breast ( $n = 5$ ) and ovarian ( $n = 3$ ) tumors as well as breast cancer cell lines SK-BR-3, 21MT-2, and H16N2 in an attempt to correlate disease with mutations. No mutations were found, although our analysis was limited to the open reading frame and did not include the putative promoter. The open reading frame was identical in C35<sup>hi</sup> cell lines and tissues as well as C35<sup>lo</sup> cell lines.

C35 is very highly conserved among six higher eukaryotic species examined (identities >77%) but does not seem to have orthologues in microbial organisms (Fig. 2). Furthermore, examination of the public sequence databases at the National Center for Biotechnology Information failed to identify homologues with identity >20% at the protein level in any species. We expanded our search to look for functional motifs and discovered a match to the catalytic site of Selenoprotein W from several species. Despite the homology to a selenoprotein, C35 is not predicted to contain a selenocysteine residue (12), which is encoded by TGA stop codon when certain RNA structures are present in the 3' untranslated region. The 3' untranslated region of C35 does not contain necessary hairpin loops that would cause selenocysteine translation. Furthermore, we have not seen evidence of elongated native C35 protein using Western blots (Fig. 4) or immunoprecipitation that would result from translation including a selenocysteine residue.

C35 contains several functionally important motifs, as predicted by the algorithms of NetPhos (13), NetNGlyc,<sup>5</sup> NetOGlyc (14–16), PIR pattern search (17), and OMIGA (Accelrys, San Diego, CA). Casein kinase II, cyclic AMP, and cyclic guanosine 3',5'-monophosphate phosphorylation sites were predicted at amino acids Tyr<sup>39</sup>, Ser<sup>76</sup>, and Ser<sup>97</sup>. Additionally, the C35 sequence EATYLELASAVKEQYPGIEI conforms to a prototypical immunoreceptor tyrosine-based

<sup>5</sup> R. Gupta, E. Jung, S. Brunak. Prediction of N-glycosylation sites in human proteins. 2004, in preparation.

Homo sapien	MSGEPGQTSVAPPPEEVEFPGSGVRIVVEYCEPCGF <b>EATYLELASAVKEQYPGIEIESRLG</b>	60
Macaca mulatta	MSGEPGQTSVAPPPEEVEFPGSGVRI <b>M</b> VVEYCEPCGF <b>EATYLELASAVKEQYPGIEIESRLG</b>	60
Mus musculus	MSGEP <b>A</b> PV <b>S</b> V <b>V</b> PP <b>P</b> GE <b>V</b> E <b>A</b> GSG <b>V</b> H <b>I</b> VVEY <b>C</b> K <b>P</b> CG <b>F</b> EATYLELASAVKE <b>E</b> Y <b>P</b> GIEIESRLG	60
Rattus norvegicus	MSGEP <b>Q</b> V <b>S</b> V <b>V</b> PP <b>P</b> GE <b>V</b> E <b>A</b> GSG <b>V</b> H <b>I</b> VVEY <b>C</b> K <b>P</b> CG <b>F</b> EATYLELAS <b>S</b> L <b>E</b> E <b>E</b> Y <b>P</b> GIEIESRLG	60
Bos taurus	MSG <b>D</b> T <b>G</b> T <b>T</b> S <b>V</b> APP <b>P</b> GE <b>T</b> E <b>F</b> P <b>H</b> G <b>V</b> RIVVEYCEPCGF <b>EATYLELASAVKEQYPGIEIESRLG</b>	60
Canis familiaris	MSGEP <b>G</b> P <b>T</b> S <b>E</b> A <b>P</b> PP <b>E</b> G <b>E</b> I <b>E</b> FPGSGVRIVVEYCEPCGF <b>EATYLELASAVKEQYPGIEIESRLG</b>	60
Xenopus tropicalis	MS----- <b>V</b> S <b>I</b> VVEYCEPCGF <b>K</b> S <b>H</b> <b>V</b> <b>E</b> L <b>E</b> L <b>A</b> S <b>A</b> V <b>L</b> <b>E</b> <b>F</b> <b>E</b> <b>D</b> V <b>T</b> <b>H</b> <b>D</b> S <b>R</b> <b>F</b> <b>G</b>	40
Homo sapien	GTGAFEIEINGQLVFSKLENGGFFPYEKDLIEAIRRASNGETLEKITNSRPP <b>CVIL</b>	115
Macaca mulatta	GTGAFEIEINGQLVFSKLENGGFFPYEKDLIEAIRRAS <b>K</b> G <b>E</b> P <b>L</b> EKITNSRPP <b>CVIL</b>	115
Mus musculus	GTGAFEIEINGQLVFSKLENGGFFPYEKDL <b>M</b> E <b>A</b> IRRASNGE <b>P</b> V <b>E</b> KITNSRPP <b>CVIL</b>	115
Rattus norvegicus	GTGAFEIEINGQLVFSKLENGGFFPYEKDL <b>M</b> E <b>A</b> IRRASNGE <b>P</b> L <b>E</b> KITNSRPP <b>CVIL</b>	115
Bos taurus	GTGAFEIEINGQLVFSKLENGGFFPYEKDLIEAIRRASNGE <b>P</b> L <b>E</b> KITNSRPP <b>CVIL</b>	115
Canis familiaris	GTGAFEIEINGQLVFSKLENGGFFPYEKDLIEAIRRASNGE <b>P</b> L <b>E</b> KITNSRPP <b>CVIL</b>	115
Xenopus tropicalis	GTGAFEIEINGQLVFSKLE <b>L</b> G <b>G</b> F <b>P</b> Y <b>A</b> KDLIEAIR <b>K</b> ASNGE <b>P</b> V <b>E</b> KITNS <b>Q</b> A <b>P</b> CVIL	95

**Figure 2.** C35 protein sequence is conserved among species. National Center for Biotechnology Information accession numbers are as follows: *Homo sapiens*, AY508814; Rhesus monkey *Macaca mulatta*, XP\_001091012; *Mus musculus*, NP\_079835; *Rattus norvegicus*, XP\_001081367; *Bos taurus*, XP\_582041; *Canis familiaris*, XP\_537653; *Xenopus tropicalis*, NP\_001015996. A predicted prenylation site (CVIL) is bolded and italicized. The immunoreceptor tyrosine-based activation consensus sequence is in bold.

activation consensus sequence comprised of the following residues: "D/E/N xx Y xx L/I<sub>x6-8</sub> Y xx L/I." Finally, the COOH-terminal amino acid sequence "CVIL" fits the prenylation motif CaaX. Based on several physical properties, such as size, flexibility, membrane buried preference, and presence of leucine as the X residue, C35 is predicted to be geranylgeranylated *in vivo* by GGTI enzyme ( $P = 0.0005$ ), resulting in the addition of 20-carbon isoprenoid moiety (18). Interestingly, the "CVIL" sequence in C35 is completely conserved among all species for which an orthologue can be found.

### Regulation of C35 Transcription

Because of the close proximity of C35 and ERBB2, and because the entire ERBB2 locus, including flanking genes, have been shown to be amplified in primary tumors, two experiments were designed to assess regulation of C35 expression in cultured cells. First, real-time PCR analysis was done using cDNA and DNA from several cancer cell lines to determine if gene amplification correlated with overexpression of C35. Figure 5 shows significant gene amplification (10- to 20-fold) and overexpression of C35 cDNA in breast tumor cell lines compared with H16N2, a nontumorigenic breast cell line with normal levels of C35 and ERBB2, and MDA-MB-231, a breast tumor cell line that does not overexpress C35 or ERBB2. In contrast, the colon carcinoma cell line Colau 267p and melanoma cell line MEL1700 up-regulate C35 transcription without gene amplification. A second experiment using luciferase-based

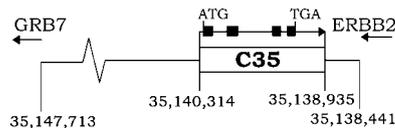
reporter assays was designed to investigate transcriptional control via the promoter region of C35 as an alternative to gene amplification. The minimal C35 promoter cloned into a luciferase reporter construct resulted in increased luciferase activity in C35-overexpressing lines. This suggests that transcription factor control contributes to increased C35 expression in the cell lines examined. It is important to note that luciferase activity is relatively high in C35<sup>+</sup> cell lines, whether or not these lines show amplification of chromosome 17q~12. It seems, therefore, that the increase in expression of C35 transcripts can be achieved through either promoter control, chromosomal amplification, or possibly some combination of each.

### Characterization of Anti-C35 Antibodies

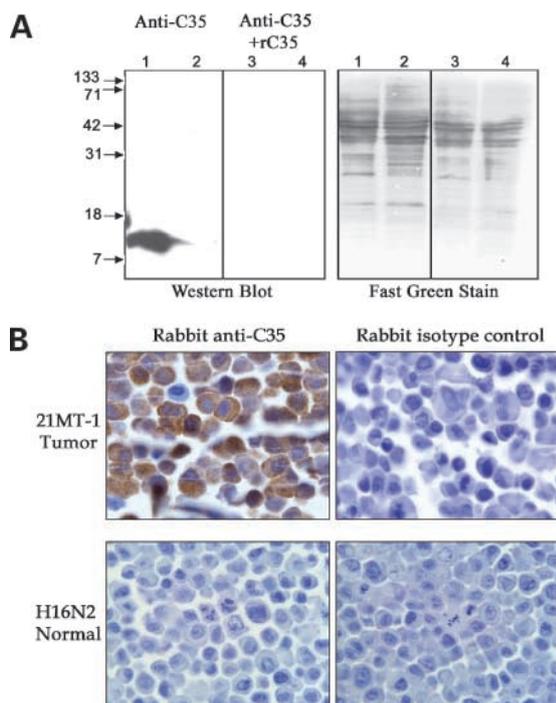
To confirm expression of the gene product of C35 in human tumors and tissues, two independent polyclonal rabbit antiserum were raised against recombinant human C35 protein. Antibodies bind one protein of expected size (12 kDa) in tumor cell lysates but not in the nontumorigenic cell line H16N2 (Fig. 4A), corresponding to transcript expression in these cell lines. Western blots were routinely done with these cell lines as well as other tumor cell lines and consistently bound this 12-kDa band. As shown in Fig. 4B, immunohistochemistry with formalin-fixed, paraffin-embedded sections further showed specific staining of the C35<sup>+</sup> tumor cell line. Importantly, independently derived antibodies reproducibly show the same pattern of reactivity in assays, including Western blot and immunohistochemistry, on a panel of normal and tumor tissues and cell lines.

### Expression of C35 and HER-2/*neu* in Breast Carcinoma

C35 and HER-2/*neu* overexpression in human breast cancer specimens was examined by immunohistochemical staining of a large panel of breast carcinomas and normal breast epithelium. Because of the proximity of the C35 and ERBB2 genes on chromosome 17, it was of particular interest to compare expression of C35 and HER-2/*neu* in each tumor sample. Figure 6A shows staining of two representative primary breast cancer samples to compare expression of C35 and HER-2/*neu*. Anti-C35 antibody staining is



**Figure 3.** Chromosomal location of C35. The C35 gene is located on chromosome 17q12, 7402 nucleotides from the start of GRB7 and 505 nucleotides from the end of ERBB2. Direction of transcription is denoted by arrows, and positions of each gene on the chromosome are identified based on Human Genome build 36.1. The full-length C35 transcript is 730 nucleotides in length, consisting of four exons (black boxes).



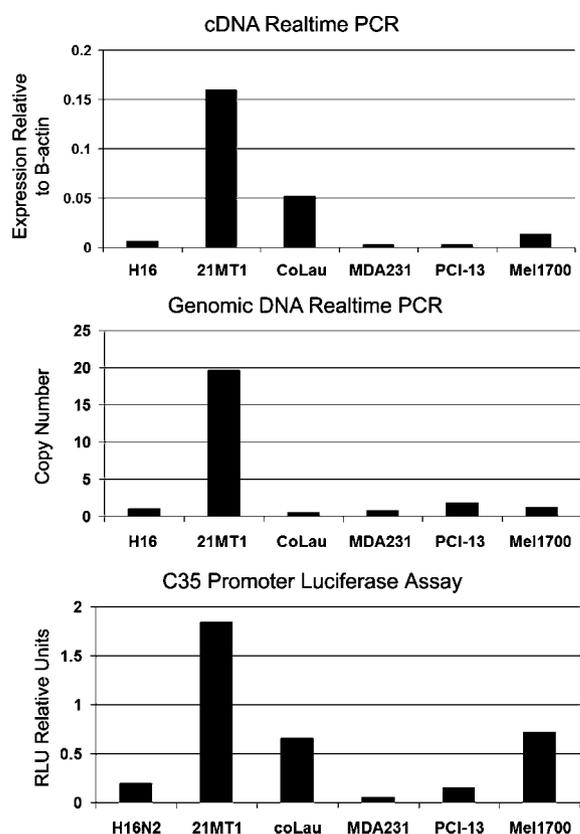
**Figure 4.** **A**, anti-C35 antibody specifically binds endogenous C35 protein in Western blot analysis. C35 protein was detected using rabbit polyclonal antiserum B78-2 in lysates of C35<sup>hi</sup> tumor 21MT-1 but not in C35<sup>lo</sup> "normal" breast cell line H16N2. Binding can be competed by preincubation of the antiserum with purified C35 protein, showing specificity of the antibody in the assay. Corresponding fast green stains of the blots show equal sample loading and transfer. Molecular weight markers (kDa) are indicated with arrows. **B**, immunohistochemistry of cell lines derived from metastatic breast carcinoma and normal breast epithelium. Sections of formalin-fixed, paraffin-embedded cell pellets were stained with polyclonal rabbit anti-C35 or isotype control rabbit IgG antibodies. Tumor stains intensely with a punctate pattern. Magnification,  $\times 100$ .

characterized by an intense punctate cytoplasmic staining pattern. Punctate staining suggests vesicular localization of the protein, consistent with the predicted membrane association of C35 via prenylation. HER-2/*neu* shows characteristic plasma membrane staining and contrasts with C35 staining throughout the cytoplasm, including internal membranes.

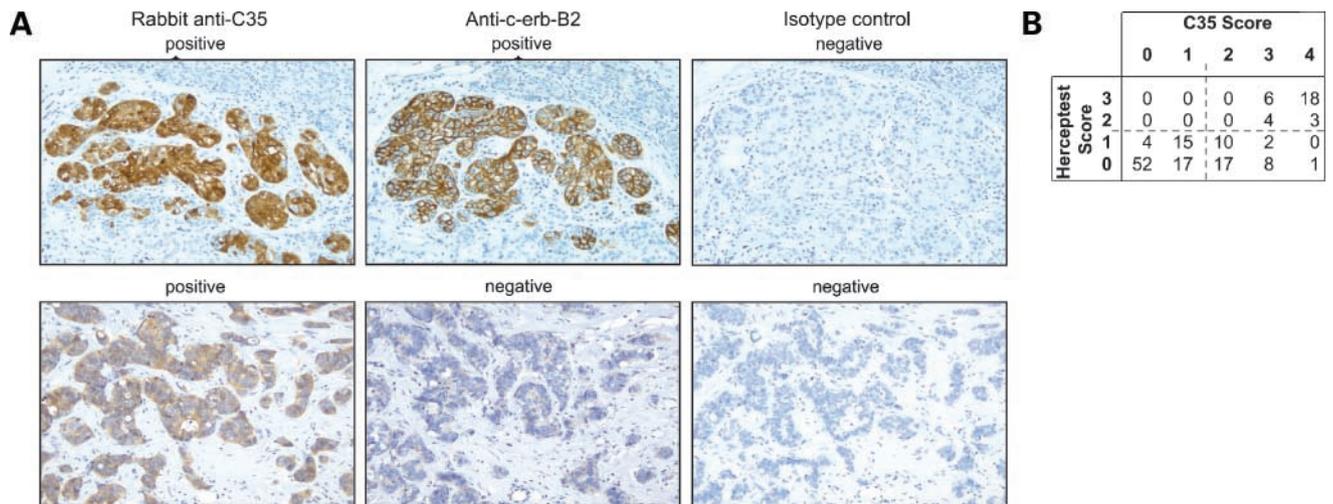
The frequency of C35 expression (Table 1) in various types of breast cancer was analyzed separately in three independent studies. Overexpression of C35 was detected in intraductal and infiltrating lobular carcinoma as well as in less prevalent forms of breast cancer, including papillary and mucinous carcinomas. Both medullary carcinoma and phyllodes sarcomas were negative for C35 expression; however, the samples sizes studied were small ( $n = 6$  and  $n = 3$ , respectively). The frequency of C35-positive tumors was similar among the three studies. Although  $\sim 40\%$  to  $50\%$  of breast carcinomas express elevated levels of C35, expression in normal breast epithelium was low. A low percentage of normal breast samples were weakly positive for C35 expression; however,  $70\%$  of the "normal" breast

specimens included in these studies were adjacent to tumor from diseased patients. Figure 7B shows that, even in the few samples with positive normal breast tissue, C35 staining in tumor cells is substantially more intense than staining of adjacent normal breast epithelial cells. Furthermore, the mean C35 scores for normal breast were significantly lower than mean scores of high-grade and total IDC as well as ILC.

Almost one half of IDCs, the most common type of breast cancer, were positive for C35 overexpression. Another notable feature of these three studies is the correlation of HER-2/*neu* and C35 overexpression in IDC. HER-2/*neu* expression was consistent with current literature (20-30% positivity in IDC). Strikingly, all HER-2/*neu*<sup>+</sup> tumors were also C35<sup>+</sup> (Fig. 6B) and, indeed, express the highest levels of C35 (median C35 score of 3.6 for HER-2/*neu*<sup>+</sup> and 2.3 for HER-2/*neu*<sup>-</sup> tumors). C35 expression also tends to positively correlate with grade and stage of IDC. Expression of C35, but not HER-2/*neu*, in grade 1 tumor and higher levels of C35 expression in HER-2/*neu*<sup>+</sup> grade 2 and 3



**Figure 5.** C35 transcript regulation. Cell lines were analyzed for abundance of C35 gene or transcript by real-time PCR to compare genomic amplification with expression of mRNA. All genomic amplification data are relative to H16N2, which is set to reflect one copy. The C35/*ERBB2* locus is amplified in 21MT-1, corresponding with overexpression of both transcripts in this line. In contrast, C35 transcript is overexpressed in Colau without amplification of the gene locus. Furthermore, luciferase activity driven by C35 promoter region mimics cDNA levels and contrasts with genomic copy number in C35<sup>+</sup> cell lines, such as Colau and Mel1700.



**Figure 6.** Immunohistochemistry of intraductal carcinoma of breast. **A**, sections of formalin-fixed, paraffin-embedded tissue were stained with polyclonal rabbit anti-C35, anti-c-erb-B2, or isotype control antibodies. *Top*, example of a C35<sup>+</sup>/ERBB2<sup>+</sup> tumor (scores of 4+ and 3+, respectively); *bottom*, example of a C35<sup>+</sup>/ERBB2<sup>-</sup> tumor (scores 2+ and 0, respectively; faint cytoplasmic stain is negative according to HercepTest guidelines). ERBB2<sup>+</sup> tumor has intense plasma membrane staining, whereas C35<sup>+</sup> tumors show intense, homogeneous, cytoplasmic staining. Magnification, ×40. **B**, comparison of immunohistochemistry scores for HER-2/*neu* and C35 in number of IDC cases. Dotted lines indicate delineation between positive and negative scores.

tumors suggests that C35 expression increases with tumor progression. Increasing C35 frequency during disease progression can also be seen when correlating with stage (18% in stage I and up to 50% in stage IV). Frequency of C35 is higher than HER-2/*neu* at all stages. In addition, in the 42 cases that had both IDC and DCIS, C35 expression in IDC and DCIS was concordant. Expression of HER-2/*neu*, in contrast, has been shown to be quite heterogeneous, especially among DCIS and IDC from the same sample (19), suggesting that antigen-negative cells may less readily escape C35-targeted than HER-2/*neu*-targeted therapies.

ILCs, the second most common type of breast cancer, also overexpress C35 at frequencies significantly higher than those seen in normal breast samples. C35 can be an important novel biomarker for ILC, especially due to lack of expression of other biomarkers, such as HER-2/*neu*, in this type of breast cancer. Lobular carcinoma has been shown in other studies to be ERBB2 negative (20), in agreement with the data presented. Another significant finding is the correlation of C35 positivity with younger ILC patients (Fig. 8). The average age of patients that score C35 positive is 52.9, whereas patients with C35-negative ILC tumors average 62.5 years. This age correlation was not seen in patients with IDC.

Besides correlation with HER-2/*neu* expression, these studies revealed other interesting features of C35 expression. The pattern of expression of C35 within a tumor sample is relatively homogeneous (virtually all of the tumor cells in a given sample stain with the same intensity). No significant correlation was found between C35 expression and tumor size. Many of the C35<sup>+</sup> samples exhibited necrosis and lymphocytic infiltration, and, although not independent prognostic factors, these features have been associated with poor clinical outcome. C35 expression also

correlates with lymph node involvement (Spearman two-tailed  $P = 0.04$ ). Grade, stage, and ERBB2 expression are other prognostic factors that can be correlated with C35 expression.

#### C35 Expression in Distant Metastases

As shown above, C35 expression is intense in metastatic cell lines 21MT-1, 21MT-2, T-47D, and SK-BR-3. Although the primary tumors were not available for comparison, C35 expression was detected in 6 of 12 distant metastases of breast cancer patients with primary infiltrating breast carcinoma; representative samples are shown in Fig. 9. The most common sites of breast metastasis are bone, lung, liver, and brain. Interestingly, four of four liver metastases were positive for C35 overexpression. C35 was also detected in distant metastases to lung ( $n = 1$ ) and skin on mid back ( $n = 1$ ), whereas absent in metastases to lymph node ( $n = 1$ ), vagina ( $n = 1$ ), chest wall ( $n = 2$ ), and carina ( $n = 1$ ). The presence of C35 in distant metastases again shows the persistence of the protein throughout progression of disease.

#### Restricted C35 Expression in Normal Human Tissue

C35 is highly expressed in breast carcinomas compared with normal breast epithelium and other normal tissues, including a broad panel of 38 normal human tissues. Three to five different samples of each tissue were analyzed, including adrenal gland, bladder, blood, bone marrow, brain cerebellum, cerebral cortex, cervix, colon, endothelium, esophagus, eye, fallopian tube, gastrointestinal, heart, kidney, lung, liver, lymph node, mesothelium, ovary, pancreas, parathyroid, peripheral nerve, pituitary, placenta, prostate, salivary gland, skin, skeletal muscle, spleen, stomach, testes, thymus, thyroid, tonsil, ureter, and uterus. Representative images are shown in Fig. 7. Strikingly, none of the normal tissues were positive for C35 expression, with

the exception of Leydig cells in testes in three of three samples and some ductal epithelium from normal breast at a low frequency and intensity, as described above. Several tumor antigens, including NY-ESO1 and members of the MAGE, BAGE, and GAGE families, display a similar tissue distribution among normal tissues and testes and have been termed cancer/testes antigens (21). Cancer/testes antigens are, however, expressed in germ cells, whereas C35 is restricted in expression to the Leydig cells.

## Discussion

Identification of phenotypic and genetic changes that are conserved throughout breast cancer progression of the disease may help to illuminate key events necessary to transform healthy cells, maintain malignancy, and facilitate development of metastases. Proteins associated with these changes may also be exploited as biomarkers useful in diagnosis, prognosis, and potentially as therapeutic targets. This report contains the first full description of a novel gene with a highly favorable profile as a biomarker.

Several C35-specific reagents were employed to determine expression of C35 using a variety of assays, including PCR and/or immunohistochemistry, which are described herein and can be used to further examine the expression of the biomarker. In addition to polyclonal antibodies described, several monoclonal antibodies have also been developed for use in immunohistochemistry, immunofluorescence, and Western blots. Results are concordant. The data presented indicate that C35 is broadly expressed in human breast cancer. Notably, expression of C35 persists throughout disease progression, from very early events in tumorigenesis (DCIS and LCIS) and through late stages of the disease (distant metastases). Both low-grade and early-stage IDCs show high levels of C35 expression, often arising before expression of ERBB2, an important prognostic biomarker for IDC. In addition, ~50% of tumors from late-stage patients and distant metastases exhibit abundant C35 expression. Many of these tumors have characteristics associated with poor prognosis (ERBB2 positivity, lymphocytic infiltration, lymph node involvement, and necrosis among all types studied). Indeed, C35 expression shows significant correlation with HER-2/*neu* positivity and

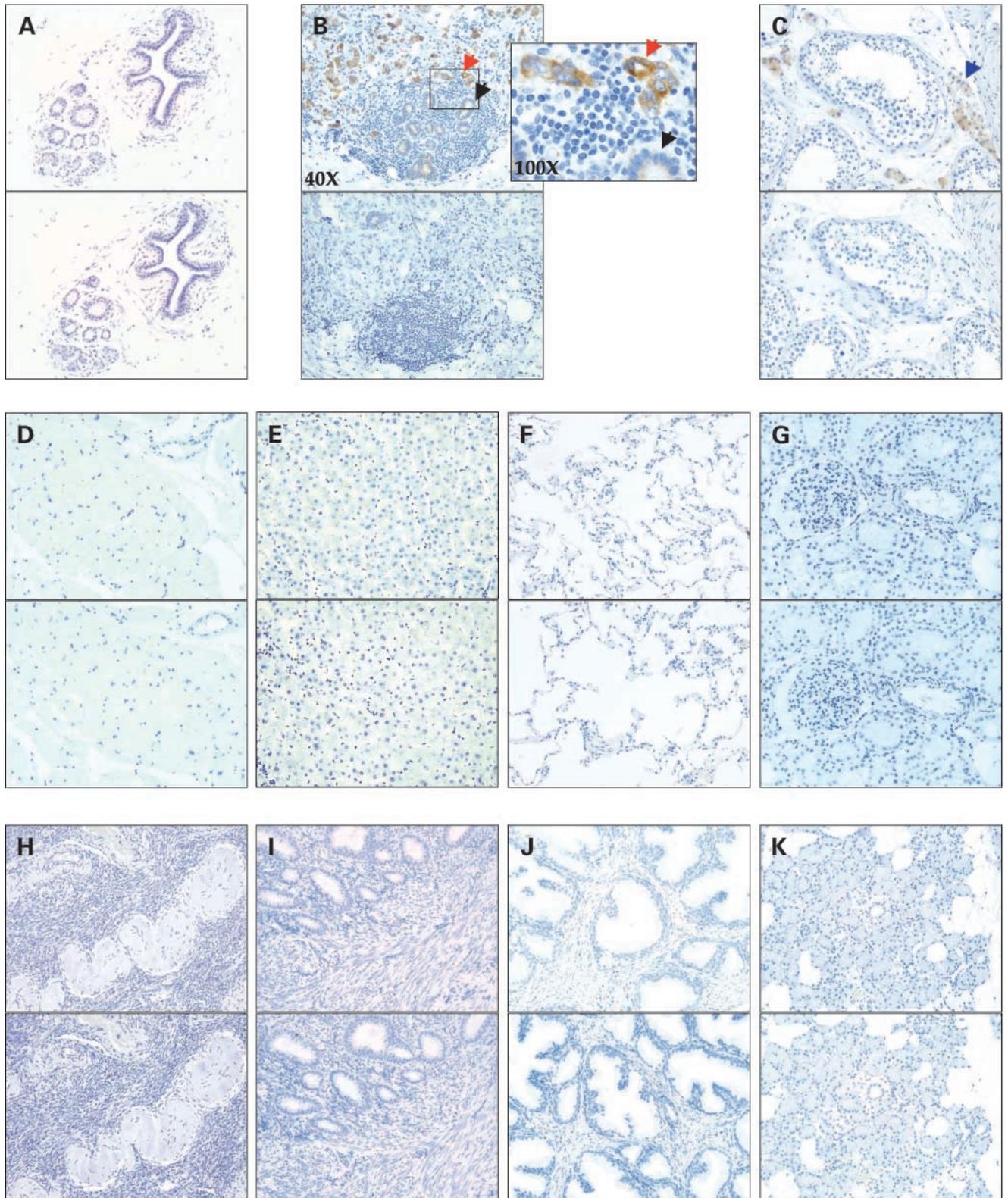
**Table 1. C35 expression in FFPE breast tissue sections was determined by immunohistochemistry**

	<i>n</i>	HER-2/ <i>neu</i> <sup>+</sup> (%)	C35 <sup>+</sup> (%)	Mean C35 reactivity	<i>P</i> (one-sample paired <i>t</i> )
<b>Intraductal carcinoma</b>					
Qualtek study	57	21	53		
AccuMax Array	30	20	40		
Asterand Array	71	20	38		
Total IDC	158	20*	44	1.46 <sup>†</sup>	<0.0001
Low grade (1)	22	0	32	0.73	
High grade (2 and 3)	35	34	66	2.03 <sup>†</sup>	<0.0001
Stage I	28	11	18	0.93	
Stage II	38	24	47	1.63 <sup>†</sup>	<0.0001
Stage III	26	19	38	1.46 <sup>†</sup>	0.0035
Stage IV	4	25	50	1.75	
<b>Lobular carcinoma</b>					
Qualtek Study	14	0	50		
AccuMax Array	4	0	25		
Asterand Array	22	5	32		
Total ILC	40	5 <sup>‡</sup>	38	1.16 <sup>†</sup>	0.0083
Papillary carcinoma: AccuMax	8	13*	50	1.13	
Medullary carcinoma: Asterand	6	0	0		
Mucinous carcinoma: Asterand	2	0	50		
<b>Normal breast tissue</b>					
Adjacent normal	40		13		
Normal breast	17		35		
Total normal breast	57		19	0.61	

\*All HER-2/*neu*<sup>+</sup> samples are also C35<sup>+</sup>.

<sup>†</sup>Significant difference compared with expression in normal breast by paired *t* test (*P* < 0.05).

<sup>‡</sup>The two HER-2/*neu*<sup>+</sup> samples are C35<sup>-</sup>.



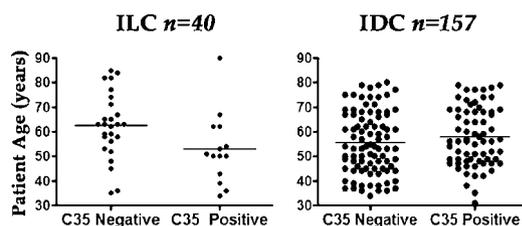
**Figure 7.** Normal human tissues stained with rabbit anti-C35 polyclonal antibodies (*top*) and control rabbit IgG (*bottom*). Magnification, 40 $\times$ . **A**, breast tissue from a non-cancer patient. **B**, breast tissue from cancer patient containing tumor (4+, *red arrows*) and adjacent morphologically "normal" epithelium (2+, *black arrows*). *Inset*, magnification,  $\times 100$ . **C**, testes, Leydig cells are C35 $^{+}$  (*blue arrows*). **D**, heart. **E**, liver (polymer detection). **F**, lung. **G**, kidney. **H**, ovary. **I**, uterus. **J**, prostate. **K**, salivary gland.

stage, both important prognostic indicators. Although prognostic value and use in early detection must be validated in further studies focusing on treatment and outcomes, C35 shows potential as a novel biomarker for breast cancer, including early detection.

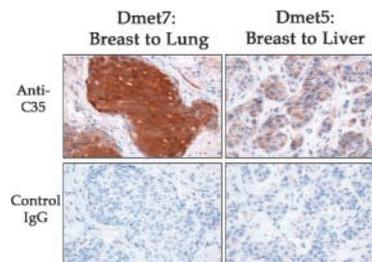
C35 can be an important novel biomarker in other breast cancers as well, especially infiltrating lobular carcinoma. Detection of ILC, the second most common form of breast cancer, is particularly problematic because these tumors may not be detected by palpitation or mammography. In addition, the presence of lymph node involvement in ILC can be missed during histologic examination due to bland, uniform appearance of the malignant cells and low mitotic rate (22). ILC typically exhibits a less aggressive phenotype (hormone receptor positivity and low frequencies of p53 abnormality and HER-2/*neu* expression). However, studies have shown that overall survival rates of ILC are similar to more aggressive IDC, which may result from poor detection due to the morphology of ILC. The data presented show an inverse correlation of C35 expression with age of ILC patients: younger patients had ILC with higher levels of C35 expression. ILC in younger patients tends to follow an aggressive course. These considerations highlight the need for a reliable biomarker, such as C35, for detection of ILC, in light of missed diagnoses and lack of other biomarkers to detect ILC.

Clear and significant correlations of C35 expression throughout the process of tumorigenesis are described, in contrast to the absence of C35 expression seen in most normal tissues. C35 expression in a wide range of breast cancer types and stages of disease significantly exceeds expression in normal tissues. Although some samples of normal breast were C35<sup>+</sup>, the low level of C35 seen in the adjacent normal breast tissue may represent predisposition to disease in these individuals. Alternatively, the positive cells may be expressing early markers of malignancy, although they do not yet seem morphologically distinct from normal breast epithelium. This would be consistent with the overexpression of C35 observed early in the progression of disease, such as DCIS, LCIS, and grade 1 IDC as well as stages I and II tumors, and underscores importance of C35 in early detection. Regulation of transcript expression may be important when comparing stage and type of cancer.

Due to juxtaposition of the C35 gene with ERBB2 and the known associations of amplification of this locus with



**Figure 8.** C35 positivity correlates with age in ILC. C35 is expressed at a higher frequency in young patients with ILC. Expression in IDC does not correlate with patient age.



**Figure 9.** C35 Expression in breast metastases. Examples of distant metastases stained with polyclonal rabbit anti-C35 or control IgG. Magnification,  $\times 40$ .

disease progression, regulation of C35 expression was assessed initially in relation to genomic amplification. The locus of chromosome 17q11~12, encompassing ERBB2 and GRB7, has been shown to be amplified in esophageal carcinomas (23), ovarian (24), endometrial (25), head and neck (26), and gastric tumors (27); gene amplification has been correlated with poor prognosis (28). A strong correlation between ERBB2 and GRB7 overexpression in breast cancer specimens has been shown (29). Because commonly used fluorescence *in situ* hybridization probes for HER-2/*neu* include the C35 gene, direct fluorescence *in situ* hybridization analysis for C35 was not done. Based on breakpoint determination of the amplified region and direct experimental evidence, we assume that C35 is amplified with the same frequency as ERBB2. However, our data suggest that genomic amplification is not the only mechanism of C35 up-regulation. Real-time PCR shows abundance of C35 transcripts in the absence of gene amplification or induction of ERBB2 transcription in some tumor cell lines. Transcriptional control that is independent of genomic copy number is evident from analysis of C35 promoter driving a higher level of luciferase reporter expression in C35<sup>hi</sup> than in C35<sup>lo</sup> cell lines. In addition, immunohistochemistry results show many examples of C35<sup>+</sup> breast tumors that do not overexpress ERBB2 proteins. Studies comparing fluorescence *in situ* hybridization and immunohistochemistry have shown that the majority of ERBB2-positive samples with immunohistochemistry scores  $\geq 2$  and 3+ result from genomic amplification, whereas cases that score 0 to 1+ are not amplified (28, 30). These data suggest that in those cases that are C35<sup>+</sup>/ERBB2<sup>-</sup>, chromosome 17q12~21 is not amplified. This hypothesis has yet to be tested. Additional transcriptional control mechanisms independent of genomic amplification suggest an important role for C35 in the process of tumorigenesis.

Although function has not been studied, analysis of predicted functional motifs of the gene product suggests a role for C35 in signal transduction. A prenylation site is predicted with a high degree of certainty at the COOH terminus. Prenylation promotes membrane association by attachment of hydrophobic moiety, thereby producing a protein with a substantially higher affinity for the membrane (18, 31). Other proteins that contain prenylation

sites include the Ras oncogene family, whose function in signal transduction cascades and transforming potential are dependent on prenylation-induced translocation to the membrane (32, 33). Lipid modification of eukaryotic proteins by prenyl transferases is required for critical signaling pathways, cell cycle progression and induction of apoptosis, cytoskeletal remodeling, and vesicular trafficking. Membrane association, predicted phosphorylation sites, and the presence of an immunoreceptor tyrosine-based activation motif suggest the possibility that C35 participates in signal transduction cascades. Immunoreceptor tyrosine-based activation sequences alone, with proper membrane localization and functional phosphorylation sites, have recently been shown to be potent oncogenes when transfected into epithelial cell lines (34). The significance of these motifs within C35 and understanding protein function may define C35's role in tumorigenesis as well as illuminate development of targeted therapies and diagnostic methods involving this novel biomarker.

Ongoing and future studies, including oncogenic potential, cellular localization, and interacting proteins, should elucidate function of the novel protein, which may lead to direct targeting, small interfering RNA, or other small molecule-based approaches. Inhibitors of prenylation have been shown to possess antitumor activities, such as slowing tumor growth (35) and promoting tumor rejection in animal models (36). There are several current clinical trials of prenyl transferase inhibitors in acute myelogenous leukemia (37), breast, colorectal, and pancreatic cancers (38). C35 may serve as a target for these therapies as well. Immunotherapy is another possibility. Preliminary data suggest that C35, although it is an overexpressed self-protein, can in fact be immunogenic in humans.<sup>6</sup> Coexpression with ERBB2 also raises the possibility of effective and specific combined therapies. Approximately 15% to 30% of IDC patients who are eligible for anti-ERBB2 therapies may also benefit from C35-directed therapies. Furthermore, C35 is often expressed in absence of ERBB2 overexpression and is overexpressed in a higher proportion of patients. This broad population also includes patients with less aggressive tumors, such as grade 1 IDC, ILC, who could be treated at earlier stages of disease, and who may, therefore, be more responsive to novel cancer-specific therapies. In patients who have progressed to late-stage disease, effective therapy must target metastases. It is important, therefore, that high levels of C35 expression are detected in distant metastases from the breast, as shown by the data presented. In limited immunohistochemical and RNA analysis of tumor cell lines and small groups of primary tumors, we have seen evidence of C35 overexpression in several other tumor types, including melanoma, colon, ovarian, pancreatic, lung, and bladder carcinomas. The frequency and intensity of expression

may, however, be lower relative to breast cancer. Large-scale immunohistochemistry studies are planned to confirm frequency and association with other prognostic factors in breast and in other adenocarcinomas.

The abundance and prevalence of C35 overexpression in tumors and the absence of expression in normal human tissues make it an attractive choice as a biomarker for the diagnosis of early-stage and late-stage cancer as well as a potential target for therapeutic intervention. Although expression of C35 is clearly linked with HER-2/*neu* amplification in some cases, C35 presents advantages as a novel biomarker in an equal number of IDC that do not overexpress HER-2/*neu*, and particularly in early stage and ILC that typically do not overexpress HER-2/*neu*. Furthermore, the two proteins are independent, and C35 can represent an additional target for therapy. Regulation and expression of C35 in DCIS, LCIS, IDC, ILC, and metastases could suggest a role for this gene in process and maintenance of malignant phenotype. Further investigation into C35 function may elucidate effective therapy at targeting this molecule to prevent and/or treat breast cancer.

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# Molecular Cancer Therapeutics

## C35 (C17orf37) is a novel tumor biomarker abundantly expressed in breast cancer

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