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A Novel Splice Variant of *HER2* With Increased Transformation Activity

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The *HER2* proto-oncogene (also known as *neu* or c-*erbB*-2) belongs to the epidermal growth factor receptor family. *HER2* is frequently amplified in human carcinomas. Gene amplification or overexpression of *HER2* has been correlated with poor prognosis in several human cancers. Point mutation in the rat *HER2* homolog, *neu*, is involved in the formation of rat neuroblastomas. However, no similar mutation in *HER2* has been found in human cancers. Here we report the identification of a novel alternative splicing form of HER2 (ΔHER2) in human cell lines. An exon 16 amino acids long in the extracellular domain was deleted in ΔHER2. Deletion mutations in the corresponding region were shown previously to be involved in the formation of mammary carcinomas in transgenic mice. In the focus-formation assay, ΔHER2 showed much stronger transformation activity than did wild-type HER2. This result suggests that the deleted 16–amino acid exon may play a regulatory role in HER2 transformation activity. *Mol. Carcinog. 23:62–68, 1998.*

Key words: HER2; receptor protein-tyrosine kinase; proto-oncogene; alternative splicing; transformation

INTRODUCTION

The rat *neu* oncogene was originally identified by a chemical mutagenesis experiment in the rat, and *HER2* is the human counterpart. The *HER2/neu* gene (also known as c-*erbB*-2) encodes a 185-kDa receptor tyrosine kinase that belongs to the epidermal growth factor receptor family [1]. A single point mutation (valine to glutamic acid) in the transmembrane domain can activate the rat *neu* proto-oncogene [2]. The same amino acid change introduced by in vitro mutagenesis into human HER2 has also been shown to activate the transformation ability [3]. No identical mutation in *HER2* has been found in human cancers [4,5]. However, overexpression and gene amplification of *HER2* is frequently observed in human cancers. Poor prognosis was found to correlate with overexpression or gene amplification of *HER2* in several cancers [6–10]. In vitro experiments demonstrated that overexpression of HER2/neu was sufficient to activate the tyrosine kinase activity in the absence of mutation or ligand stimulation [11,12]. Transgenic mice that overexpressed normal rat neu protein (the rat HER2 homolog) in the mammary epithelium develop mammary carcinomas with high penetrance [13]. The formation of tumors in these transgenic mice correlates with de novo mutations of the *neu* transgene that activates its tyrosine kinase activity in the tumors. A novel class of small deletions in the extracellular domain was found in these tumors. These deletion mutations were shown to activate the in vitro transformation activities of neu by enhancing dimerization and tyrosine kinase activities [14]. Here we report the identification of an alternatively spliced mRNA in human cancer cell lines. This alternatively spliced *HER2* variant appeared as smaller bands in our reverse transcription (RT)–polymerase chain reaction (PCR) analysis of *HER2* expression in human cancer cell lines. Strikingly, the alternative splicing form of *HER2* (referred to hereafter as *ΔHER2*) has one exon deleted from the coding sequence, and this deleted exon overlaps with the deletions in the corresponding region of the rat *neu* transgenes. The similarity in the predicted protein structure of ΔHER2 and the deletion mutants in transgenic mice suggests that ΔHER2 may be an activated form of HER2 and therefore may play an important role in human cancers.

MATERIALS AND METHODS

Oligonucleotide Primers Sequences

The primers used were NP-1 (5′-CAT GCC CAT CTG GAA GTT TC-3′ (nt 2004–2023)), NP-2 (5′-GCT CCA CCA GCT CCG TTT CCT G-3′ (nt 2269–2248)), NP-5 (5′-ATG CCA GCC TTG CCC CAT CAA CTG C-3′ (nt 2040–2064)), and NP-6 (5′-AGA CCA CCC CCA AGA CCA CGA CCA G-3′ (nt 2185–2161)). The nucleotide sequence numbers are based on GenBank sequence X03363.

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Abbreviations: RT, reverse transcription; PCR, polymerase chain reaction; MAPK, mitogen-activated protein kinase.

RT-PCR Analysis of Cancer Cell Lines

RNA was extracted from cancer cell lines with TRIZOL (Life Technologies, Gaithersburg, MD). The RT reaction was performed with Superscript II reverse transcriptase by following the manufacturer's protocol (Life Technologies). Primers NP-2 and NP-5 were used for the RT-PCR analysis (Figure 1B). The expected sizes of RT-PCR products were 206 and 158 bp for wild-type *HER2* and *ΔHER2,* respectively. The RT-PCRs were performed in the presence of 1 μCi of [^{32}P]dCTP in a reaction volume of 50 μL. Ten to twenty microliters of the PCR products was separated on a 6% polyacrylamide gel. The gel was dried before exposure to X-OMAT film (Kodak, Rochester, NY) for autoradiography.

Sequencing of RT-PCR Products

The RT-PCR bands from the dried polyacrylamide gel were cut out, and the DNA was eluted by boiling in H_2O for 2 min. Aliquots of the DNA solutions were reamplified with the same primers, and the PCR products were directly sequenced with a Cyclist sequencing kit (Stratagene, La Jolla, CA) by using ^{32}P end-labeled primers or an [^{35}S]dATP incorporation protocol.

PCR and Sequencing of Genomic DNA

High-molecular-weight DNA (0.5–1 μg) was amplified with primers NP-5 and NP-6 (Figure 1B) by using the Expand Long Template PCR System (Boehringer Mannheim, Indianapolis, IN) and following the manufacturer's protocol. The PCR products were separated on both 1% and 3% agarose gels to detect large and small PCR products. Only one PCR product of 6 kb was observed, and it was excised from the 1% agarose gel and purified with Qiaex II (Qiagen, Valencia, CA). The purified DNA samples were sequenced manually with ^{32}P end-labeled primers by using the Cyclist sequencing kit or with an automatic sequencer (Applied Biosystems, Inc., Ramsey, NJ) in the core sequencing facility of M. D. Anderson Cancer Center.

Plasmid Construction

The wild-type human *HER2* cDNA coding region was released from psv2erb2 (kindly provided by Dr. T. Yamamoto) by HindIII digestion and subcloned into the plasmid Bluescript (Stratagene) for mutagenesis. The RT-PCR fragment of *ΔHER2* from the cell line BT-474 was amplified with primers NP-1 and NP-2. The 218-bp RT-PCR product was digested with SphI and AatII and subcloned into Bluescript/HER2 to generate a *ΔHER2* clone. Point-mutated *HER2* in Bluescript was generated by digesting the vector LTR1erb-Glu (kindly provided by Dr. P. P. Di Fiore) [3] with SphI and NdeI and was subcloned into the same sites in Bluescript/HER2. HindIII fragments from these *HER2* clones in Bluescript were cut out and subcloned into pcDNA3 (Invitrogen, Carlsbad, CA). The wild-type HER2, *ΔHER2,* and point-mutated *HER2* pcDNA3 clones were named CMV-HER2-WT, CMV-HER2-Δ, and CMV-HER2-Glu, respectively. The EcoRI fragment from CMV-HER2-Δ was cut out and used to replace the corresponding region in LTR1erb-Glu to create LTR-HER2-Δ.

Elk-GAL4 Luciferase Assay

CHO cells were transfected with 0.5 μg of Elk-GAL4, 6 μg of GAL4-LUC (kindly provided by Dr. C. J. Der) [15], 1.5 μg of RSVβ-gal, and 2 μg of cytomegalovirus promoter–driven *HER2* expression vectors by the calcium phosphate method. Luciferase activities were normalized to β-galactosidase activities. The same experiment was repeated with CMV β-gal as a normalization control. The experiment was also repeated in NIH/3T3 cells by following the same procedures. The cells were harvested 36–48 h after transfection and assayed for luciferase activity by using the Luciferase Assay System (Promega Corp., Madison, WI).

Western Blotting

COS-7 cells were resuspended at 10^7 cells/mL of Dulbecco's modified Eagle's medium/F-12 medium with 10% fetal bovine serum. Four hundred microliters of cell suspension was mixed with DNA at room temperature and pulsed at 260 V and 960 μF in a 0.4-cm cuvette by using Gene Pulser equipment (Bio-Rad Labs., Hercules, CA). After transfection, the COS-7 cells were allowed to grow for 24–36 h before being harvested. Fifty micrograms of lysate was separated on a 6% sodium dodecyl sulfate–polyacrylamide gel, transferred to a nitrocellulose membrane by using the Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad Labs.), and blotted with PY20 anti-phosphotyrosine antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The same membrane was stripped by following the procedures recommended in the ECL kit (Amersham Corp.) and reprobed with c-neu antibody Ab3 (Oncogene Science, Cambridge, MA). Signals were detected by the enhanced chemiluminescence method (Amersham Corp.).

Focus Formation Assay

NIH/3T3 cells (2-3x10^5) were plated in 6-cm tissue-culture dishes and grown overnight. Ten micrograms of test plasmids (or 2 μg of test plasmids plus 8 μg of Bluescript plasmid in a lower-concentration experiment) was transfected by the calcium phosphate protocol. Two or three days after transfection, the cells were split 1:25 into 10-cm tissue-culture dishes and were grown in Dulbecco's modified Eagle's medium/F-12 medium supplemented with 10% fetal bovine serum. After 14–21 d, the cells were stained and fixed for focus counting. The experiment was repeated three times. Two independent preparations of plasmids were used to avoid bias due to variation in the quality of different DNA preparations.

RESULTS

While studying the expression of *HER2* in human cancer cell lines by RT-PCR analysis, we unexpectedly, obtained additional smaller bands (Figure 1A). The same results were also observed with other human cancer cell lines, including SK-OV3, BT-474, and SK-BR3 (data not shown). The region amplified by this set of primers contains the transmembrane and juxtamembrane regions of HER2. To determine whether these RT-PCR products came from nonspecific amplification, the bands were excised from the gel, reamplified, and then sequenced directly. The sequences of the small products matched the predicted sequence of *HER2* except for a deletion of 48 bp in the extracellular domain (Figure 1B). The 48-bp deletion created an in-frame deletion of 16 amino acids. To determine whether the small products resulted from mutation in the genomic DNA in these cancer cell lines, primers NP-5 and NP-6 (Figure 1B) were used to amplify genomic DNA extracted from the cell lines A431, MDA-MB-361, and HBL-100. The PCR products from the three cell lines were directly sequenced. By comparing the DNA sequences, it became clear that the deleted 48 bp was one exon (Figure 1C). The mRNA variant with the 48-bp deletion will be referred to hereafter as *ΔHER2*. We propose that *ΔHER2* came from alternative mRNA splicing because the sequences of the RT-PCR products of *ΔHER2* were consistent with the predicted alternative splicing products. Furthermore, no point mutation in the splice site junctions was observed (data not shown). This type of point mutation has been shown to account for most mRNA mis-splicing or exon skipping in human diseases [16]. The primers NP-5 and NP-6 amplified PCR products of the same size from the genomic DNA of all three cell lines. The sequences of the PCR products corresponding to the 48-bp exon and the exon-intron junctions were identical. Running different percentage agarose gels revealed no additional PCR products indicating deletion in genomic DNA. This evidence suggests that *ΔHER2* is an alternative splicing variant rather than a product of mutation in the genomic DNA.

It is known that tyrosine phosphorylation of the *HER2*-encoded receptor and its tyrosine kinase activity are important indicators of its activity. To determine the tyrosine phosphorylation activity of different forms of HER2, *HER2* cDNA expression vectors were transfected into COS-7 cells, and western blotting was performed with an antibody against phosphorylated tyrosine residues (PY20) 24 h after transfection (Figure 2). *ΔHER2* and the point-mutated *HER2* produced a much stronger tyrosine phosphorylation signal in the predicted position of the HER2 protein than did the wild-type HER2. The same nitrocellulose membrane was stripped and reprobed with anti-HER2–specific antibody c-neu Ab-3, and the expression levels of HER2 in the COS-7 cells were comparable. It is clear that the transfection of *HER2* caused tyrosine phosphorylation of a 185-kDa protein and that ΔHER2 had much stronger activity than did wild-type HER2 in this assay.

The ras-dependent pathway is one of the downstream signaling pathways of HER2/neu. To examine whether ΔHER2 was able to activate its downstream signals, activation of the ras pathway was tested by transient transfection of CHO cells and NIH/3T3 cells with an Elk-GAL4/GAL4-luc reporter system [15]. The expression of luciferase in this system depends on the phosphorylation of the Elk-GAL4 fusion protein by the mitogen-activated protein kinases (MAPKs). The ras pathways activate the MAPKs, which in turn activate luciferase activity in this system. The results from this assay indicated that ΔHER2 was indeed more active in activating MAPKs than was wild-type HER2. ΔHER2 activated MAPKs sixfold more strongly than did wild-type HER2 in CHO cells (Figure 3). The same experiments were repeated with NIH/3T3 cells, and the results were essentially the same (data not shown). Interestingly, the activity of ΔHER2 in this assay was comparable to that of the point-mutated HER2, which is known to be a potent transforming oncogene.

One of the most important properties of activated HER2/neu is its strong transformation ability. To test the transforming ability of ΔHER2, focus formation of NIH/3T3 cells was assayed. *ΔHER2* was subcloned into an expression vector driven by the murine leukemia virus long terminal repeat promoter. The transformation ability of this construct was compared to that of a wild-type *HER2* construct and a point-mutated *HER2* construct in the same expression vector. Wild-type *HER2* produced no significant increase in the number of foci above the levels of the negative control (Bluescript plasmid) in multiple experiments, whereas *ΔHER2* and point-mutated *HER2* induced foci efficiently under these conditions (Table 1). ΔHER2 formed as many foci as point-mutated HER2 did, but the foci formation was slower and the foci were generally slightly smaller (Figure 4). The experiments were repeated three times, and two independent preparations of plasmids were used. There was variation in the number of foci formed between different sets of experiments, but the difference between wild-type HER2 and ΔHER2 was so dramatic that we conclude that the deletion in the extracellular domain in ΔHER2 strongly enhanced the transformation ability of HER2.

DISCUSSION

Here we reported the identification of a deletion form of the human *HER2* mRNA in cancer cell lines by RT-PCR analysis. The human *HER2* genomic sequence flanking the deleted region was partially determined by direct sequencing of PCR products. Our results suggested that ΔHER2 was generated by alternative splicing of mRNA rather than mutation. Re-

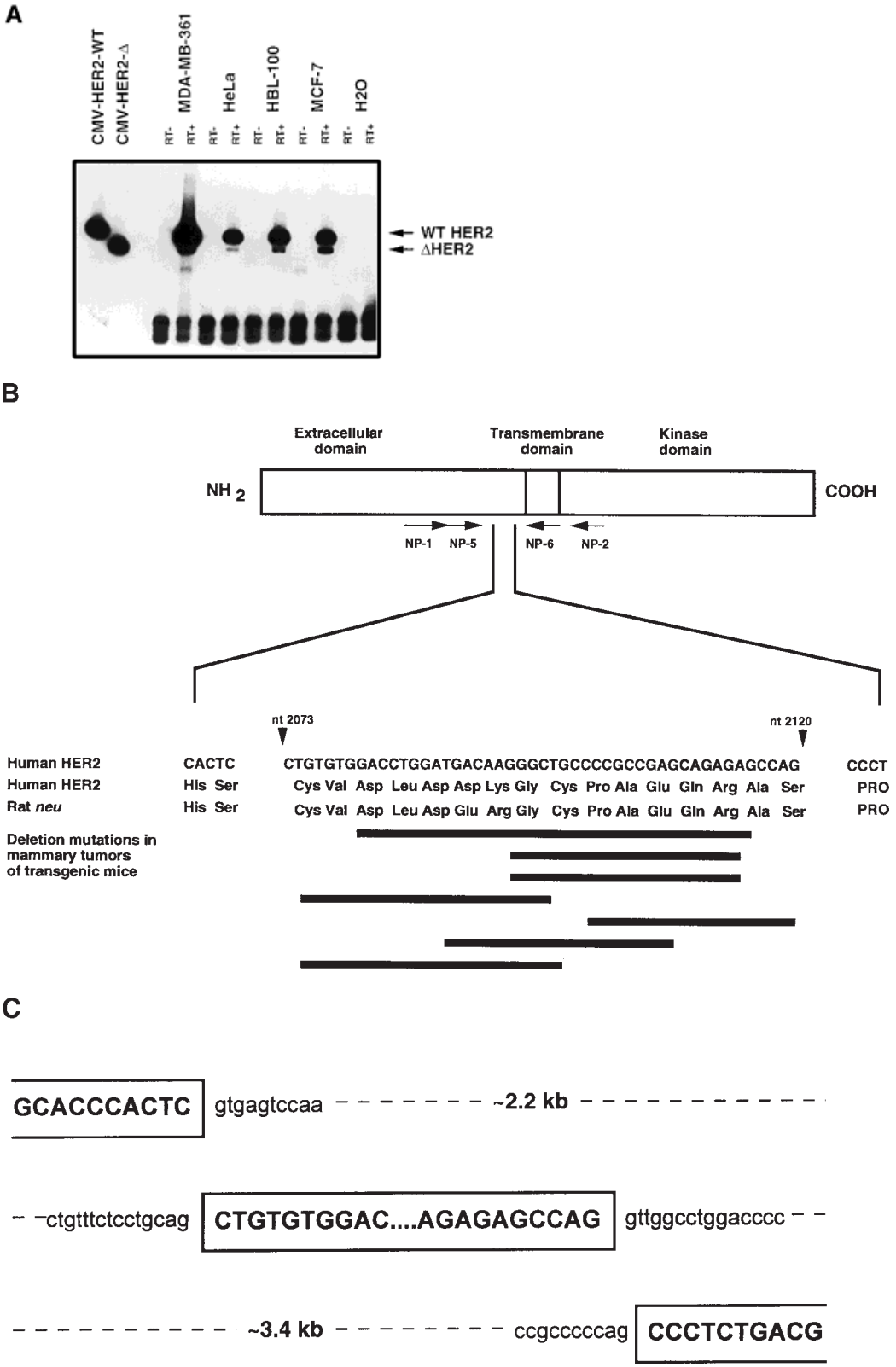

Figure 1. Detection of a novel *HER2* mRNA (*ΔHER2*) in human cancer cell lines. (A) RT-PCR was performed with primers NP-2 and NP-5 in the presence of [^{32}P]dCTP. The upper bands are the wild-type *HER2* RT-PCR products, and the lower bands are the alternative splicing products. The reactions were performed in the presence (RT+) or absence (RT–) of reverse transcriptase. The leftmost two lanes contain cloned cDNAs that served as positive and size controls for the RT-PCR reactions. CMV-HER2-WT is a wild-type *HER2* cDNA expression vector, and CMV-HER2-Δ was engineered by replacing the wild-type *HER2*

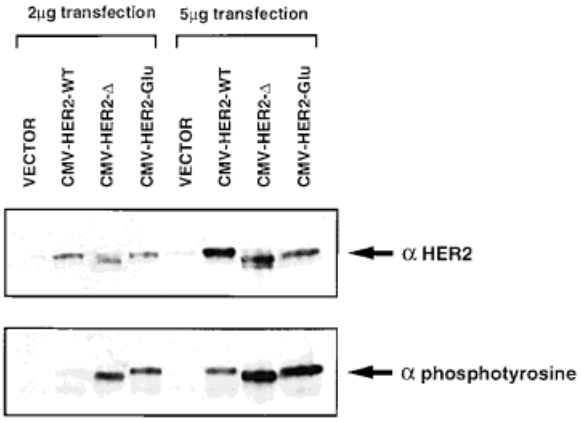

Figure 2. Expression of ΔHER2 was associated with strong tyrosine phosphorylation. *HER2* cDNAs were subcloned into pcDNA3 and transfected into COS-7 cell by electroporation. The transfected cells were harvested 24 h later and analyzed by western blotting with anti-phosphotyrosine antibody PY-20. PY-20 was then stripped, and the same membrane was reprobed with anti-HER2 antibody c-neu Ab-3. Note that the ΔHER2 protein ran slightly faster than the wild-type HER2 protein. CMV-HER2-Glu is the point-mutated human *HER2* cDNA, which is a well-characterized oncogenic form of *HER2*.

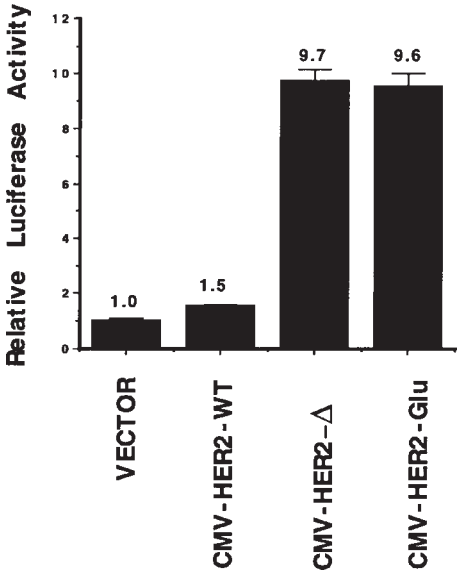

Figure 3. ΔHER2 was a potent MAPK activator. CHO cells were transfected with 0.5 μg of Elk-GAL4, 1.5 μg of RSVβ-gal, 6 μg of GAL4-LUC, and 2 μg of *HER2* cDNAs in pcDNA3. The luciferase activity reflects the phosphorylation of the Elk-GAL4 fusion protein by the MAPKs. The result showed here is from one experiment. Transfections of each *HER2* vector was triplicated. Luciferase activities are normalized to β-galactosidase activities. The activity of the empty vector was set to one. The results indicated that ΔHER2 activated MAPK activity as strongly as did point-mutated HER2 and about sixfold more strongly than did wild-type HER2.

moval of the same 48 bp deleted in ΔHER2 from the wild-type HER2 cDNA activated its transformation ability in NIH/3T3 cells.

Mutations in rat *neu* gene have been shown to be responsible for the formation of tumors in animal experiments, and clinical studies have clearly demonstrated the importance of *HER2* in human cancers. *HER2* overexpression or gene amplification is observed in 20–30% of human breast and ovarian cancers and is associated with poor prognosis in these patients [6,7]. The rat *neu* proto-oncogene can be activated by a single point mutation in the transmembrane domain or by deletion of the entire extracellular ligand binding domain [11,17]. However, no equivalent mutations have been found in human cancers. The absence of point mutations in *HER2* in human cancers could be explained by the fact that the equivalent amino acid change from valine to glutamic acid require a 2-bp change in human *HER2* (GTT→GAG) and therefore requires two point mutations. On the other hand, in the rat *neu* gene, a 1-bp change is sufficient for the same amino acid change (GTG→GAG). It has been suggested that the probability of having both point mutations required for the valine to glutamic acid change in the human *HER2* gene is too low to be observed in human cancers.

Transgenic mouse models have been established to study the role of neu in mammary carcinogenesis. Expression of the wild-type rat *neu* cDNA transgene induces mammary carcinomas in the animals. Small de novo deletions in the neu extracellular domain were observed very frequently in these tumors [14]. The sequences deleted in these tumors all overlap with the deleted exon in human *ΔHER2* reported here. Because the rat *neu* cDNA construct used in the transgenic mouse experiment did not contain introns, the deletions in the mice tumors represent mutations instead of alternative splicing. The investigators who performed these experiments also studied the activation mechanism of the mutant *neu* transgenes and demonstrated that the deleted region contains critical cysteine residues. Removal of these cysteine residues in the rat *neu* cDNA by deletions strongly enhances the receptor dimerization, and, as a result, the tyrosine kinase activity of neu is increased. In vitro mutagenesis by the same group showed that disrupting these cysteine residues in the wild-type rat *neu* gene by point mutations is sufficient to activate the transformation ability of *neu* [18]. In the human *ΔHER2* gene reported here, the deleted exon contains two of these conserved cysteines residues. It is possible that the strong transformation activity of ΔHER2 we observed was also due to enhanced receptor dimerization and increased tyrosine kinase activity.

There is a previous report of another alternative splicing form of human *HER2*. This alternative splic-

sequence with the RT-PCR product from *ΔHER2* mRNA. (B) RT-PCR products from BT-474 and MDA-MB-361 were directly sequenced, and the difference between wild-type *HER2* and *ΔHER2* was determined to be the absence of one 48-bp exon (nt 2073–2120) in *ΔHER2*. The solid bars below the *HER2* sequence indicated the published deletion regions in the tumors of *neu* transgenic mice [14]. (C) The exon and intron junctions in human *HER2* in the cell line HBL-100. Capital letters indicates exon sequences, and lowercase letters indicate intron sequences.

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