Alternative HER-2/neu Oncogene Products and Their Roles in Carcinogenesis

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CERTIFICATE OF APPROVAL

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Chapter 1

Thesis Introduction

The HER-2/neu oncogene is the human homologue of the *neu* proto-oncogene, which was initially identified in rat neuroblastomas induced by the mutagen N-ethyl-nitrosylurea (Coussens et al., 1985; Yamamoto et al., 1986). The human HER-2/neu gene is located on the long arm of chromosome 17 and spans approximately 78 kilobases (kb) with at least 28 exons (Coussens et al., 1985). Like HER-2, the *neu* gene encodes a single membrane-spanning receptor tyrosine kinase, p185neu, with extensive homology to the epidermal growth factor receptor (EGF-R). Unlike HER-2, *neu* requires an activating mutation for its transforming capacity in the rat: a single base-pair mutation that results in a Valine to Glutamic acid substitution at amino acid residue 664 (V664E), which resides within the transmembrane region of the molecule (Bargmann et al., 1986). Introduction of a charged residue at this position was subsequently shown to confer constitutive tyrosine kinase activation upon rat *neu* (Bargmann & Weinberg, 1988). The orthologous mutation (V659E) in the human HER-2/neu gene product, p185HER-2, requires two base pair changes and has not been found in human tissues (Hynes & Stern, 1994; Suda et al., 1990).

The HER-2 gene is transcribed into a 4.5 kb HER-2 messenger RNA (mRNA) and 185 kilodalton (kDa) protein product, p185HER-2 (Coussens et al., 1985), which is developmentally expressed and is essential (Lee et al., 1995), yet its normal function is entirely unknown (Tzahar & Yarden, 1998). HER-2/neu gene disruption causes early embryonic lethality (E9-10) in mice, and is required for normal development of neural tissues and ventricular trabeculation of the myocardium (Lee et al., 1995). HER-2/neu is expressed abundantly in many human fetal tissues and displays ubiquitous low levels of expression in adult human tissues (Press et al., 1990), with the exception of some hematopoetic cell lineages that do not express detectable levels of HER-2 (Hynes & Stern, 1994).

Class I Receptor Tyrosine Kinases

The HER-2/neu gene product is a member of the EGF-R family (class I) of receptor tyrosine kinases (RTK). There are four homologous members of the EGF-R family: HER-1 (erbB-1, EGF-R), HER-2 (neu, c-erbB-2), HER-3 (c-erbB-3), and HER-4 (Hynes & Stern, 1994). EGF-R is the prototype of class I RTKs with the following structural features (Ullrich & Schlessinger, 1990). The extracellular domain (ECD) confers ligand binding and is divided into four subdomains (I-IV)(van der Geer et al., 1994). Subdomains I and III are implicated in low- and high-affinity receptor-ligand interactions, respectively, since sites within these regions were shown to be involved in EGF-R bivalent ligand binding (Lax et al., 1988; Tzahar et al., 1997; Summerfield et al., 1996; Lax et al., 1991). Subdomains II and IV are rich in cysteine residues with a highly-conserved distribution, and are thought to provide the structural features of the ligand-binding pocket (Lax et al., 1991). The ECD is connected to the intracellular domain by a single transmembrane domain (van der Geer et al., 1994). The cytoplasmic domain is composed of a highly conserved tyrosine kinase catalytic site that is responsible for receptor transphosphorylation and substrate phosphorylation (Ullrich & Schlessinger, 1990). In addition, the carboxyl-terminus contains several tyrosine autophosphorylation sites of unique sequence that mediate activation state-dependent signaling via second messenger association with phosphorylated sites (van der Geer et al., 1994; Ullrich & Schlessinger, 1990). Among known intracellular second messengers of EGF-R signaling are the phosphotyrosine binding (PTB)-domain proteins, phosphatidyl inositol-3 kinase (PI-3 K) and phospholipase Cγ (PLCγ), and src-homology domain-containing (SH2) proteins, such as Shc and Grb2, which mediate signaling through the mitogen-activated protein (MAP) kinase cascade (Cohen et al., 1995; Seger & Krebs, 1995; Ben-Levy et al., 1994; Kavanaugh et al., 1994; Ming et al., 1994).

Activation of Class I RTKs

In addition to sharing structural features, the EGF-R family members are activated by a common mechanism. Activation is initiated by receptor dimerization that is typically induced by ligand binding to cognate receptor monomers with one-to-one stoichiometry (Tzahar et al., 1997; Heldin & Ostman, 1996; van der Geer et al., 1994; Woltjer et al., 1992; Ullrich & Schlessinger, 1990). Receptor dimerization is an absolute requirement for activation of the tyrosine kinase catalytic domain (Weiss & Schlessinger, 1998; Qian et al., 1995). Catalytic activation is thought to be mediated by a conformational change in receptor monomers induced by ligand binding and subsequent dimer formation (Weiss & Schlessinger, 1998; Tzahar et al., 1997; Dougall et al., 1994). Dimers can be homomeric or heteromeric among all four members of the EGF-R family (Earp et al., 1995; Carraway & Cantley, 1994). Indeed, all ten possible dimers have been shown to occur in a hierarchical nature, with p185HER-2 as the overall preferred dimer partner (Graus-Porta et al., 1997; Tzahar et al., 1996).

Despite strong similarities, several distinctions exist between EGF-R family members. EGF-R (HER-1) and HER-4 are activated by their cognate ligands in the conventional manner (Beerli & Hynes, 1996; Riese et al., 1996). HER-3, however, shares homologous structure and specifically binds NDFs, inducing rapid dimerization, but is kinase inactive (Zhang et al., 1996; Wallasch et al., 1995; Guy et al., 1994). Conversely, HER-2 is an orphan receptor to which no ligand that binds directly with high affinity has yet been identified, despite intense investigations (Tzahar & Yarden, 1998; Pinkas-Kramarski et al., 1998, 1996; Peles & Yarden, 1993; Peles et al., 1993, 1992). Thus, without a known activation-inducing ligand, HER-2 homodimer function in signaling has been difficult to study. To date, the signaling activation of HER-2 has mainly been investigated with regard to heterodimer signaling, as HER-2 has been shown to undergo

heterodimerization in a ligand-inducible manner with cognate co-receptors, especially the kinase-inactive HER-3 (Tzahar et al., 1996; Zhang et al., 1996; Earp et al., 1995; Wallasch et al., 1995).

EGF-like Ligands

Regulation of EGF-R family member activity is complex, owing to the discovery of several EGF family ligands and the fact that these receptors form homodimers as well as heterodimers. All known mammalian EGF family ligands function to stimulate dimerization of their cognate receptors (Tzahar & Yarden, 1998). The motif responsible for activation is an EGF domain, comprising six cysteines and a few other essential residues with a characteristic, conserved distribution (Groenen et al., 1994). It has recently been shown that EGF-R family ligands are bivalent (Tzahar et al., 1997). The amino-terminus of their EGF-like domain binds to subdomain III of the receptor with high affinity and narrow specificity (Tzahar et al., 1997; Summerfield et al., 1996). The carboxyl-terminal region of the ligand binds to a second receptor, the dimer partner or co-receptor, with low affinity and broad specificity to a site that may be within subdomain I of the co-receptor (Woltjer et al., 1992; Lax et al., 1991, 1990).

Specificity for their cognate receptors serve to classify ligands into three groups. Epidermal growth factor (EGF), transforming growth factor alpha (TGFα), and heparin-binding EGF-like growth factor (HB-EGF) activate EGF-R with nM affinity (Higashiyama et al., 1991; Savage et al., 1972). Betacellulin (BTC) is a ligand for both EGF-R and HER-4 (Beerli & Hynes, 1996; Riese et al., 1996). Neu differentiation factors (NDF), or heregulins, exist in several alternatively spliced forms and bind to both HER-3 and HER-4 with high affinity (Tzahar et al., 1997; Peles & Yarden, 1993; Plowman et al., 1993). Recently, neuregulin-3 was identified as a HER-4 ligand in neural tissue, where this receptor is endogenously expressed (Zhang et al., 1997). In addition to

ligand-mediated stimulation of RTKs, their activation can also occur in response to stress, such as hyperosmotic shock and UV irradiation by an unknown mechanism (Weiss et al., 1997).

Although there is a growing number of known activating ligands, few natural inhibitors or antagonists have been described. Among them, only one, the Drosophila Argos, is a class I RTK antagonist (Howes et al., 1998). Argos is structurally related to the activating EGF-R family ligands in that it contains a single EGF domain and is secreted (van de Poll et al., 1997; Schweitzer et al., 1995). Argos is postulated to exert its antagonistic effect by competing with activating ligands for binding, thereby inhibiting EGF-R dimerization (Schnepp et al., 1998). Angiopoietin-2 is the other known extracellular inhibitor of a mammalian RTK, the Tie-2 endothelial receptor, to which it binds, but fails to induce activation (Maisonpierre et al., 1997). Much effort has been invested in understanding the mechanisms underlying ligand binding and activation in order to facilitate designing inhibitors, since antagonists of EGF-R family members and other class I RTKs have potential therapeutic utility (Groenen et al., 1994).

Oncogenic Activity of HER-2

Gene transfer studies have shown HER-2/neu to be the single most potent oncogene in its unaltered form. Overexpression alone of the wild-type HER-2/neu cDNA, encoding p185, confers malignant transformation (DiFiore et al., 1987; Hudziak et al., 1987). This occurs in the apparent absence of an activating ligand (Dougall et al., 1994; Rodrigues & Park, 1994; Segatto et al., 1988). In addition, transgenic mice engineered to overexpress "wild-type" human p185HER-2 develop metastatic mammary tumors (Guy et al., 1992; Suda et al., 1990). This is in contrast to any other RTK and to rat p185neu, which require either the presence of ligand or activating mutations to induce a malignant phenotype (Cohen et al., 1996; Dougall et al., 1994; Weiner et al., 1989; Bargmann et al., 1986; Hung et al., 1986).

EGF-R family members, especially p185HER-2, have been implicated in many human cancers (Hynes & Stern, 1994). HER-2/neu overexpression has been found in several human adenocarcinomas, and, most notably, in up to 30% of human breast and ovarian carcinomas, where its overexpression confers poor prognosis (Ross et al., 1998; Press et al., 1990; Slamon et al., 1989, 1988, 1987). HER-2 expression may have an important role in many other human tumors of epithelial origin as well, including colon, prostate, non-small cell lung cancer, cervical, esophageal, and oral squamous cell carcinoma (Cohen et al., 1989; Arai et al., 1997; Kern et al., 1990; Hynes & Stern, 1994; Mitra et al., 1994).

Significant evidence exists that HER-2/neu is a potent oncogene in human adenocarcinomas. In both breast and ovarian cancer, where HER-2 has been most widely studied, increased p185HER-2 expression levels resulting from HER-2 gene amplification predict a decreased survival time in a dose-dependent manner(Slamon et al., 1989, 1988, 1987). p185HER-2 overexpression in breast cancer predicts a 9.5-fold increased relative risk for tumor recurrence (Press et al., 1990). In addition, HER-2/neu overexpressing breast and ovarian tumors display decreased responsiveness to adjuvant chemotherapy and to endocrine therapy, such as tamoxifen (Pegram et al., 1997; Felip et al., 1995; Benz et al., 1993).

Further evidence of the oncogenic effects of HER-2 have been gained by abrogating HER-2 expression at the surface of human tumor cells, the overwhelming effect of which is tumor regression. Downregulation of p185HER-2 expression by several approaches has confirmed this. Conditional HER-2 expression using a tetracycline-repressible promoter can regulate tumorigenesis (Fruendieb et al., 1997; Baasner et al., 1996; Kistner et al., 1996); ribozymes (Juhl et al., 1997) and antisense oligonucleotides (Pegues & Stromberg, 1997) have been used to selectively degrade HER-2 mRNA; coexpression of adenovirus EIA has been used to decrease HER-2 translation and

synthesis (Yu et al., 1993); single-chain intracellular antibodies that bind nascent HER-2 monomers and trap it in the endoplasmic reticulum have been effective (Deshane et al., 1995; Beerli et al., 1994); forced expression of dominant negative receptor isoforms have been used to bind the receptor at the cell surface and block its dimerization and activation (Qian et al, 1994); and extracellular domain-specific monoclonal antibodies have been used to downregulate p185HER-2 cell surface expression by inducing internalization (Rodriguez et al., 1993; Hudziak et al, 1989).

Mechanisms for Enhanced Potency

The current model describing the oncogenic potency of HER-2 states that HER-2 gene amplification leads to increased transcription of the 4.5 kb mRNA encoding p185HER-2 and results in cell surface overexpression. Overexpression favors dimerization and, thus, tyrosine kinase catalytic activation (Dougall et al., 1994; Samanta et al., 1994; Lonardo et al., 1990). Activation by autophosphorylation and transphosphorylation leads to enhanced signaling for growth and tumor cell proliferation, and further may induce an invasive, anchorage-independent phenotype (Xu et al., 1997; Yu et al., 1994; Yu et al., 1993).

Possible mechanisms underlying the oncogenic potency of p185HER-2 involve its high intrinsic kinase activity in auto- and transphosphorylation. In addition, p185HER-2 displays enhanced ligand-independent homodimerization and activation with increased cell surface expression (DiFiore et al., 1987; Hudziak et al., 1987). Although the possibility of an as-yet-uncharacterized activating ligand is not ruled out, this seems unlikely since heterologous HER-2 overexpression induces a transformed phenotype, which appears to be ligand-independent (Segatto et al., 1988; Hudziak et al., 1987).

Another possible mechanism for the oncogenic potency of HER-2 is by heterodimerization. As the preferred heterodimer partner, HER-2 displays synergistic transforming activity when co-expressed with either EGF-R (Kokai et al., 1989) or HER-3 (Alimandi et al., 1995; Wallasch et al., 1995). The preferential interaction with p185HER-2 is suggested to occur through a low-affinity, promiscuous ligand-binding site located within its subdomain I, which interacts with a broad specificity binding site within the carboxyl-terminus of the ligand's EGF-like domain (Tzahar et al., 1997). According to this model, ligand bivalence promotes heterodimer interaction by a high affinity interaction with its primary receptor and a promiscuous, low-affinity interaction with preference for p185HER-2 (Graus-Porta, 1997; Tzahar et al., 1997, 1996). Heterodimerization enhances RTK signaling and proliferative effects by deceleration of ligand dissociation from heteromeric receptor complexes and by enhanced recycling to the cell surface, rather than receptor degradation (Lenferink et al., 1998; Huang et al., 1990). This latter effect is thought to occur by facilitating dissociation of receptor heterodimer-ligand complexes in the early endosome, as opposed to the late endosome or lysosome, where receptor constituents are destined to the degradation pathway.

Furthermore, HER-2 has been postulated to increase the invasive phenotype via interaction of p185HER-2 with beta-catenin. Activated p185HER-2 has been shown to phosphorylate the carboxyl-terminus of beta-catenin and induce its dissociation from E-cadherin in adhesion complexes (Ochiai et al., 1994) which mediate the tight cell-to-cell junctions of epithelial cell layers. Dissociation of beta-catenin from adhesion complexes is thought to weaken cell-to-cell adhesions (Adams et al., 1996). Studies conducted in breast carcinoma cells using forced expression of dominant-negative amino-terminal deletion mutants of beta-catenin in an invasion assay support the hypothesis that this HER-2-mediated phosphorylation event might initiate an invasive phenotype (Shibata et al., 1996). Further, it has been proposed that this event may

facilitate progression to anchorage-independence and metastasis by allowing HER-2 overexpressing tumor cells to escape the epithelial monolayer.

Mechanisms for Overexpression of HER-2 in Human Cancer

Accumulated evidence supports a role in human tumorigenesis for overexpression, as the primary aberrance, of the unaltered HER-2/neu gene. Proposed mechanisms for increased HER-2 expression include gene amplification, transcriptional, and post-translational upregulation (Kraus et al., 1987).

Gene Amplification

HER-2 gene amplification has been detected in 20-25% of human breast and ovarian cancers, and likely represents a major mechanism for HER-2 overexpression in human tumors (Zhang et al., 1989). Increase in gene copy number occurs by an unknown mechanism resulting in apparent tandem duplication of the unaltered gene.

Enhanced mRNA Levels

HER-2 mRNA accumulation is observed in conjunction with gene amplification, but to a greater extent than can be accounted for by increased gene copy number alone, in many cell lines that exhibit HER-2 overexpression (Hynes & Stern, 1994). In several breast and ovarian carcinoma cell lines, including SKBR3, BT474, and SKOV-3, the HER-2 transcript is expressed at up to 140-fold the level detected in nontumorigenic cells (Hollywood & Hurst, 1993; Tyson et al., 1991). Although both transcriptional and post-transcriptional mechanisms for mRNA accumulation are possible (Kornilova et al., 1992), upregulation of HER-2 mRNA has only been reported to occur as a result of increased transcription initiation rate (Hollywood & Hurst, 1993).

Clinical Utility of the HER-2 Oncogene

The high incidence of p185HER-2 overexpression and its association with poor prognosis suggest that it should be an important target of cancer therapy. Moreover, since it is expressed at the cell surface at abnormally high levels in tumor as opposed to normal cells, it provides a therapeutic target at the molecular level. Thus, p185HER-2 overexpression has led to efforts at developing anti-cancer therapeutics that target the ECD (Pelgram et al., 1998; Baselga et al., 1996; Rodriguez et al., 1993; Hudziak et al, 1989).

One example is a humanized recombinant monoclonal antibody, Herceptin (Genentech), which is targeted toward the ECD (Baselga et al., 1996). Herceptin recognizes an epitope that is located between residues 529 and 625 (Michael Shepard, personal communication with JPA). Herceptin causes down-regulation of p185HER-2 at the cell surface and may induce its degradation (Baselga et al., 1996; Hudziak et al., 1987). In addition, Heregulin induces antibody-dependent cellular cytotoxicity (ADCC) by activating the complement pathway and immune cells *in vivo* (Pegram et al., 1997). Herceptin treatment alone showed a 12-16% response rate in patients with HER-2-overexpressing metastatic breast cancer in phase II (Baselga et al., 1996) and phase III clinical trials (Pegram et al., 1997), and it has been shown to increase effectiveness of adjuvant chemotherapy in HER-2 overexpressing cancers by two-fold, eliciting a 24% response rate (Pegram et al., 1998). Herceptin has recently received Federal Drug Administration (FDA) approval for use as an anti-cancer therapeutic in breast carcinoma patients. However, Herceptin is less effective than was originally predicted, given that 30% of breast cancers overexpress p185HER-2. Since 100% of patients entered in the Herceptin clinical trials had HER-2 overexpressing breast cancer, this level of response translates to only about 4% of the total breast cancer patient population.

One explanation for the low level of effectiveness that was addressed in the Herceptin clinical trials is the presence of a soluble HER-2 ECD that is proteolytically cleaved at the surface of tumor cells, shedding the target that Herceptin is directed against into the serum (Lin & Clinton, 1990). The presence of the soluble ECD can be detected in breast cancer patients, occurring at serum concentrations of up to 5 μg/ml, and correlates with tumor load in metastatic disease (Brodowicz et al., 1997a; Molina et al., 1996; Kandl et al., 1994). This soluble HER-2 ECD contains the recognition epitope of Herceptin and neutralizes the antibody's cytotoxic effects (Baselga et al., 1996; Brodowicz et al., 1997b). Indeed, it was reported that the titer of circulating Herceptin quickly fell below therapeutic levels in patients with detectable HER-2 ECD in serum (Pelgram et al., 1998; Baselga et al., 1996). Moreover, Herceptin response inversely correlated with serum levels of the soluble HER-2 ECD: patients whose tumors progressed during the trials demonstrated a concomitant rise in serum HER-2 ECD levels (Pelgram et al., 1998).

Another potential method of specifically targeting HER-2 overexpressing tumor cells is ligand- or antibody-mediated toxin delivery to these cells (Rodriguez et al., 1993). Unfortunately, anti-HER-2 therapeutic design based on the current model, stating that overexpression of p185HER-2 is the primary aberrance, has proven surprisingly limited in effectiveness against HER-2 overexpressing breast cancers.

HER-2 as a Prognostic Marker

The clinical utility of HER-2 as a prognostic marker, based on the expression level of p185HER-2 in tumor tissues, is limited. Numerous clinical trials have been conducted and, cumulatively, it has been determined that HER-2 overexpression predicts poor prognosis only in lymph node positive disease (Singleton & Strickler, 1992). Thus, lymph node status remains a better prognostic

indicator (Hynes & Stern, 1994; Tandon et al., 1989). There remains a need for identification of an early tumor marker that will be useful in node-negative disease.

Alternative HER-2 gene products

HER-2 function has mainly been studied based on biochemical and functional studies of the p185HER-2 product. Alternative mRNA transcripts of the HER-2 gene have been reported, although their functions have not been determined. A truncated transcript of 2.3 kb has been identified in a gastric carcinoma cell line, MKN7, was subsequently cloned from cDNA libraries of two HER-2-overexpressing breast carcinoma cell lines, BT474 and SKBR3, and was also detected in seven other carcinoma cell lines by the ribonuclease protection assay (Scott et al., 1993). Although the protein product of this transcript is predicted to be secreted, since it lacks the transmembrane-anchoring domain, it was detected only in the endoplasmic reticulum of cells. The 2.3 kb truncated transcript results from read-through of a consensus splice donor site of an intron located 5' to the transmembrane domain-encoding exon. This intron contains a consensus polyadenylation signal. This process of alternative transcript generation is termed internal polyadenylation.

Alternative transcripts of the HER-3 gene have also been identified. Four alternative HER-3 transcripts, detected in several cell lines by ribonuclease protection assay, result from intron retention with internal polyadenylation, similar to the mechanism proposed for generation of the 2.3 kb alternative HER-2 transcript (Katoh et al., 1993; Scott et al. 1993). Furthermore, they were shown to encode stable protein products when transfected into Chinese hamster ovary (CHO) cells as expression cDNA (Lee & Maihle, 1998). However, these alternative HER-3 gene products have not yet been ascribed a function.

Characterization of Alternative Forms of HER-2/neu Oncogene Expression

This thesis describes the structure and function of alternative products of the HER-2 gene. The overlying hypothesis is that these alternative products are involved in the normal and malignant function of the HER-2. First, I will describe a 4.8 kb alternative transcript and the characterization of its unique translation product. Next, I will discuss the structure and proposed function of an 8 kb HER-2 mRNA. Finally, I will summarize the implications of a 95 kDa proteolytic product of p185HER-2 in human cancer.

A novel alternative transcript of the HER-2 gene is described in chapter 2. This unique transcript of 4.8 kb (or, possibly, 2.6 kb) results from retention of intron 8 and encodes a novel protein product that acts as a ligand for the p185HER-2 receptor itself. The alternative HER-2 transcript described in this work encodes a stable secreted protein that is detectable in media from cells expressing the cDNA. It is unique in several respects. It results from internal intron retention without internal polyadenylation. It specifies a HER-2 protein with a novel C-terminal sequence encoded by intron 8. Most importantly, it binds the p185HER-2 receptor and exerts an antagonistic effect.

Another alternative HER-2/neu transcript of 8 kb has been reported in the HER-2-overexpressing ovarian carcinoma cell line, SKOV-3 (Jones et al., 1994; Karlan et al., 1994; Lichtenstein et al., 1990). This transcript appears to represent the major HER-2 transcript in these cells (i.e., it is two- to three-fold more abundant than the 4.5 kb mRNA). It has been proposed that the transcript results from a gene rearrangement in SKOV-3. Yet, despite the fact that the alternative 8 kb HER-2 transcript had not been characterized, SKOV-3 have been used repeatedly as a model for HER-2-driven ovarian cancer. Chapter 4 of this work describes the characterization of the alternative 8 kb HER-2 transcript.

Finally, since the discovery of the soluble HER-2 ECD in 1990 (Lin & Clinton, 1991; Zabrecky et al., 1991), its function has been studied mostly on a clinical level, as a potential prognostic indicator. However, the presence of the ECD as a proteolytic product suggests the concomitant presence of a constitutively active kinase remnant, relieved of the negative constraints of its ECD on receptor dimerization. We have investigated this hypothesis in breast tumor cell lines and primary tissues. Chapter 5 describes the characterization of this species, p95.

My work supports the overlying hypothesis that disruption of the normal regulation of HER-2 gene expression is the primary aberrance leading to HER-2-mediated transformation. Moreover, alternative HER-2 gene products may be involved in tumor development and progression. Defining the role of alternative products within the normal expression array of the HER-2 gene and in tumor models might help elucidate the discrepancies of the current model.

Chapter 2

The HER-2/neu oncogene encodes an antagonistic ligand for p185HER-2

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by

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ABSTRACT

HER-2/neu (erbB-2) encodes a 185 kDa orphan receptor tyrosine kinase (RTK) that transactivates HER (erbB-B) family members as the preferred heterodimer partner and displays potent oncogenic activity. Here we show that, in addition to p185HER-2, the HER-2 gene encodes a novel secreted protein, p68, that binds to p185HER-2 at the cell surface with a KD of ~14 nM. The p68 ligand is the product of an alternative HER-2 transcript that retains intron 8. This alternative transcript specifies 340 residues identical to subdomains I and II from the extracellular domain of p185HER-2 followed by a unique C-terminal sequence of 79 amino acids encoded by intron 8. The p68 mRNA is expressed in normal human fetal kidney and liver, but is expressed at significantly reduced levels relative to p185HER-2 in carcinoma cells that contain an amplified HER-2 gene. Binding of p68 does not activate, but rather inhibits p185 dimers and arrests the anchorage-independent growth of transformed cells that overexpress p185HER-2. The results presented here suggest that the HER-2 gene encodes its own antagonistic ligand, which we have named Herstatin.

INTRODUCTION

The HER-2/neu (erbB-2) oncogene encodes a receptor-like tyrosine kinase, p185HER-2, that has been extensively investigated because of its role in several human carcinomas and in mammalian development (Hynes & Stern, 1994; Dougall et al., 1994; Tzahar & Yarden, 1998). The function of the HER-2 gene has been examined mainly by the structure and biochemical properties of the 185 kDa protein product of the 4.5 kb transcript (Coussens et al., 1985; Yamamoto et al., 1986). P185HER-2 shares a common structural organization with other EGF receptor (EGFR) family members and consists of an extracellular domain (ECD), a single transmembrane segment, and a cytoplasmic tyrosine kinase domain. Dimerization of receptor tyrosine kinases (RTKs), which is typically induced by ligand binding, is required for their activation and subsequent steps in signal transduction (Heldin & Ostman, 1996). Although p185HER-2 is highly homologous to the EGFR, no ligand that directly binds with high affinity to p185 has yet been identified (Hynes & Stern, 1994; Dougall et al., 1994; Tzahar & Yarden, 1998). The signaling activity of HER-2 may be mediated through heterodimerization with other ligand-binding members of the EGFR family (Carraway & Cantley, 1994; Earp et al., 1995; Qian et al., 1995). Recent studies have suggested that EGF-like ligands bind to p185HER-2 through a promiscuous, very low affinity binding site that may recruit p185 into a heteromeric complex (Tzahar et al., 1997; Klapper et al., 1997). According to this model dimerization is mediated by bivalent EGF-like ligands that have a high affinity site that binds to the direct receptor, in subdomain III, and a second low affinity site with broad specificity that may bind to subdomain I in the ECD. Preference for interaction with the low affinity promiscuous binding site in p185HER-2 may explain its status as the preferred dimer partner (Tzahar et al., 1996; Graus-Porta et al., 1997) and its exceptional oncogenic potency (Kokai et al., 1989; Alimandi et al., 1995; Wallasch et al., 1995). Elevated basal kinase activity and constitutive dimerization, in the apparent absence of ligand, may also contribute to the oncogenic activity of p185HER-2 (Lonardo et al., 1990).

The most common mechanism by which HER-2 transforms cells is by overexpression of normal p185HER-2 in the apparent absence of a direct binding ligand (DiFiore et al., 1987; Hudziak et al., 1987). Overexpression of p185HER-2 with no evidence of mutations occurs in several human adenocarcinomas (Hynes & Stern, 1994; Dougall et al., 1994; Slamon et al., 1987). Importantly, elevated p185HER-2 in 25-30% of breast cancers predicts significantly lower survival rates and shorter time to relapse (Slamon et al., 1987, 1989), and systemic administration of antibodies against the ECD of p185HER-2 can increase the time to recurrence in a subset of patients with metastatic breast cancer that overexpress p185HER-2 (Baselga et al., 1996).

Here we describe the first example of a naturally occurring HER-2 ligand, p68ECDIIIa, encoded by the HER-2 gene itself. We present evidence that p68 ECDIIIa is an antagonistic ligand since it inhibits HER-2 dimers and the anchorage-independent growth of cells which overexpress HER-2. An antagonistic ligand, which is expressed in normal fetal tissue, could down-regulate p185 during normal development, may provide a selective pressure for p185 overexpression in human cancers, and could potentially be used as a therapeutic against cancers that are driven by HER-2 overexpression.

MATERIALS AND METHODS

Cell culture. All cell lines were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT) and 0.05% gentamycin. Media and additives were from GIBCO BRL (Gaithersburg, MD) unless indicated. Human breast and ovarian carcinoma cell lines were obtained from American Type Culture Collection (Rockville, MD). Ovarian surface epithelial cells of limited life span, IOSEVAN, were obtained from Dr. Karin Rodland at OHSU, Portland, OR. NIH-3T3 parental and a cell line stably transfected with HER-2, designated 17-3-1, were provided by Applied Biotechnology, and the NIH-3T3 cells transfected with *src* 527 were provided by Dr. Brian Druker of OHSU. Transfected cells were maintained in 0.4 mg/ml G418

(Geneticin). The HEK-293 human embryonic kidney cell line was obtained from the Vollum Institute core culture facility.

Anchorage-independent growth assays. About 1,000 cells suspended in DMEM with 10% fetal bovine serum containing 0.3% Difco Agar, were plated onto a 0.5 ml layer of media containing 0.5% agar in 12 well plates. Colonies containing at least 50 cells were counted at 21 days for SKOV-3 cells and at 14 days for 17-3-1 cells or NIH- *src* transformed cells.

Antibodies. Anti-ECDIIIa antisera were produced by Cocalico Biologicals, Inc. (Reamstown, PA) by injection of two rabbits with purified polyhistidine-tagged ECDIIIa peptide. Polyclonal anti-neu(N) was produced against a peptide identical to amino acid residues 151-165 of p185HER-2 (Lin & Clinton, 1991). Polyclonal anti-neu(C) was made against a peptide identical to the last 15 residues of the carboxyl-terminus of p185HER-2 (Lin et al., 1990). Monoclonal anti-phosphotyrosine antibody was purchased from Sigma.

Polymerase chain reaction and primer sets Templates were amplified in a Perkin Elmer GeneAmp PCR System 2400 (Perkin Elmer Cetus, Emeryville, CA) using the Expand High Fidelity PCR System (Boerhinger Mannheim) with 1X High Fidelity PCR buffer. All primers were obtained from GIBCO BRL (Life Technologies). Numbering of nucleotide and amino acid residues is according to the HER-2 cDNA sequence reported by Coussens et al. (1985). The HER-2 extracellular domain was targeted for amplification from an SKOV-3 cDNA library (Origene Technologies, Inc.) using a forward primer (A) identical to nucleotides (nt) 142-161 of HER-2 cDNA (5'-TGAGCACCATGGAGCTGGC-3'), which spans the initiation codon (underlined) and a reverse primer (B) (5'-TCCGGCAGAAATGCCAGGCTCC-3'), which is complementary to HER-2 exon sequence at nt 1265-1286. Thirty cycles of 94° C for 30", 58° C for 45", and 68° C for 3 min were used to amplify the targeted sequence.

Northern blot analysis. RNA was extracted from cells in 15 cm plates using TriReagent (Molecular Research Center, Inc., Cincinnati, OH), according to the manufacturer's protocol. For Northern blot analysis, 2.5 μg mRNA were electrophoresed in a 0.8% formalin

agarose gel and transferred to BrightStar membrane (Ambion) in 10xSSC. The membrane was prehybridized for 2 hrs at 65° C in Northern Max Prehybridization Solution (Ambion) and hybridized with ~107 cpm of an ECDIIIa-specific riboprobe at 65° C for about 15 hrs, washed, and subjected to phorphorimager analysis (Molecular Dynamics).

Ribonuclease protection assay. A template for antisense RNA probe synthesis was constructed by PCR amplification of a 389 bp sequence spanning the entire ECDIIIa insert sequence and containing adjacent 5'HER-2 exon sequence using a forward primer that is identical to HER-2 cDNA sequence at nt 1131-1152 and a reverse primer (5'-GCACGGATCCATAGCAGACTGAG GAGG-3') which contains a 3' BamH1 site and is complementary to the sequence spanning the 3' splice site of the ECDIIIa sequence. The PCR product was digested with BamH1, cloned into pBluescript SK (Stratagene), and sequenced. An antisense RNA probe complimentary to the entire ECDIIIa sequence and to 87 nt of HER-2 exon sequence 5' to the insert was transcribed from linearized template using (α-^{32}P)CTP and the T7/SP6 Riboprobe Synthesis System (Promega, Madison, WI). RNA hybrids were prepared, digested with RNaseA (Boerhinger Mannheim) and RNase T1 (Life Technologies), denatured, and electrophoresed in a 5% polyacrylamide/urea gel as described (25).

Purification of polyhistidine-tagged ECDIIIa C-terminal peptide and full length ECDIIIa protein. The ECDIIIa sequence encoded by the retained intron was amplified from a cDNA library and cloned into the pET30a vector, which encodes 6 histidine residues at the amino-terminus of the expressed protein (Novagen, Madison, WI). The resulting expression vector, pET-ECDIIIa, expressed in strain BL21 was induced with 0.1 mM IPTG and the soluble cell lysate was absorbed onto Ni-NTA agarose as per manufacturer's instructions (Novagen). The His-tagged ECDIIIa protein was eluted in buffer with 250 mM imidazole and was estimated to be approximately 90% pure by Coomassie Blue staining of gels.

For expression of the full length ECDIIIa protein, which corresponds to p68, a cDNA expression vector was constructed by subcloning the 1.3 kb Nco1-EcoR1 fragment of HER-2

cDNA (nt 149-1456) into pEt30a with an N-terminal 6xHis tag, and by replacing the 160bp Bh1-BglII fragment (nt 1075-1235) with the 435 bp BH1-BglII fragment containing the 274 bp ECDIIIa insert sequence cloned from a cDNA library. The BL21 strain of bacteria was doubly transformed with the pET-68ECDIIIa expression plasmid and with a thioredoxin expression plasmid. Expression was inducted with 0.1 mM IPTG at room termperature for 3 hours. Soluble bacterial extracts were absorbed to Ni-NTA agarose, which was extensively washed with 20mM Tris pH 8.0 containing 300 mM NaCl and 100 mM imidazole. The ECDIIIa protein, expressed as a 50 kDa protein, was eluted with Tris buffer containing 1.5 M imidazole, 0.5 mM NaCl, and 1% Chaps and then dialyzed extensively against 50 mM Tris pH 9.5 with 2 mM DTT and stored under nitrogen at 4° C. The p50ECDIIIa protein was estimated at 70% purity in Coomassie Blue stained gels.

Ligand binding and crosslinking analyses. Recombinant p50ECDIIIa, purified from bacteria, was labelled with ^{125}I using Bolton Hunter Reagent (ICN Pharmaceuticals) per manufacturer's instructions. The specific activity was about 4 x 104 c.p.m./pmol. Increasing amounts of radiolabelled p50 ECDIIIa, in the presence and absence of 100-fold excess unlabeled p50, were added in binding buffer (DMEM with 1% bovine serum albumin, BSA) to about 105 17-3-1 cells or parental NIH-3T3 cells in monolayer cultures at room temperature for 1 hr. The monolayers were washed extensively in binding buffer, extracted, and the radioactivity was quantitated. Chemical crosslinking of ligand to 17-3-1 cells was performed by adding radiolabelled p50ECDIIIa in the presence and absence of 100-fold excess unlabeled p50 for 1 hr at room temperature. The chemical crosslinking reagent bis(sulfosuccinimidyl)suberate (BS^3) was then added to a concentration of 1mM for 30 minutes at room temperature, the cells were washed with PBS, lysed, and immunoprecipitated with antibodies specific for p185HER-2 (anti-neu(C).

Preparation of conditioned media (CM). Confluent monolayers of cells (~107 cells) in 15 cm plates were washed three times with PBS and then incubated for 24 hrs with 12 ml of

serum-free DMEM. The CM was clarified by centrifugation at 13,000 x g for 20 min and was concentrated 100-fold using an Amicon filter that retains proteins of >30,000 daltons.

Western blotting. Proteins were electroblotted from SDS- polyacrylamide gels onto nitrocellulose (Trans-blot, BioRad). The membranes were blocked with 5% nonfat dry milk or with 5% BSA when anti-phosphotyrosine antibody was used. Blots were incubated with primary antibody, washed with TBS-Tween (Tris-buffered saline containing 0.05% Tween), and then incubated for 40 min with goat anti-rabbit antibody or goat anti-mouse secondary antibody conjugated to horseradish peroxidase (Bio-Rad) as described. The blots were developed with chemiluminescent reagent (Pierce) and exposed to film.

RESULTS

A novel 274 bp sequence is inserted at nucleotide 1171 in HER-2 mRNA. The polymerase chain reaction (PCR) was employed to investigate HER-2 mRNA diversity within the ECD coding sequence. A cDNA library from SKOV-3 ovarian carcinoma cells (Tyson et al., 1991) was examined using a forward primer specific for exon 1 (Tal et al., 1987), which is identical to nucleotides 142-161, and a reverse primer complementary to nucleotides 1265-1286 in exon 9 (Scott et al., 1993). A product of ~1420 nt, determined to be HER-2-specific by Southern blotting (data not shown) was approximately 270 nt larger than expected (Coussens et al., 1985). The PCR product was subcloned and the nucleotide sequence was determined. The normal HER-2 coding sequence was present beginning with the 5' primer sequence and continued uninterrupted through nucleotide 1171. At this position, a 274 nucleotide insertion was found, followed by the expected coding sequence, including the 3' primer sequence. Analysis of the predicted protein product shows that the first 340 amino acid residues, starting with the initiator methionine and signal sequence, are identical to p185 HER-2 (Coussens et al., 1985) and are followed by a 79 amino acid extension and a termination codon encoded by the 274 nucleotide insertion (Fig. 1). Inspection of the novel 79 amino acid sequence encoded by the inserted nucleotide sequence shows

a consensus N-linked glycosylation site (underlined) and a proline content of 19% (Fig. 1). The inserted sequence is designated ECDIIIa since it is located at the boundary between subdomains II and III in the extracellular domain of p185HER-2 (Lax et al., 1988). Therefore, the protein encoded by the alternative transcript is expected to be a truncated, secreted protein consisting of subdomains I and II of p185HER-2 and a novel 79 residue C-terminus. Comparison of the inserted nucleotides and their predicted amino acid sequence with sequences in Genbank showed no obvious homologies.

Examination of the 5' and 3' junctions of the divergent sequence revealed consensus splice donor and acceptor sites (Sharp & Burge, 1997) and include a pyrimidine tract and potential branchpoint adenine residues near the 3'end of the insert sequence (Fig. 1). PCR analysis of genomic DNA indicates that the 274 nucleotides are contiguous with HER-2 exonic sequence (Doherty et al., manuscript in preparation) suggesting that the inserted sequence is intron 8 based on the location of intron 8 in the homologous EGFR and HER-3 genes (Lee & Maihle, 1998).

Alternative transcripts containing the ECDIIIa sequence are expressed in human fetal kidney and liver. A Northern blot was conducted to examine whether an alternative transcript, which contains the ECDIIIa sequence, is expressed in normal human tissue. PolyA+ mRNA from a variety of human fetal tissues prepared as a Northern blot was hybridized with a radiolabeled probe specific for the unique ECDIIIa sequence. A 4.8 kb mRNA was detected in kidney and a 2.6 kb transcript was detected in liver (Fig. 2). Additionally, the 4.8 kb ECDIIIa transcript was detected in the human embryonic kidney cell line, HEK-293. The 4.8 kb transcript likely corresponds to the full length 4.5 kb transcript with the 274 nucleotide intron sequence and the 2.6 kb transcript may correspond to the previously described 2.3 kb alternative transcript (Yamamoto et al., 1986; Scott et al., 1993) with the retained intron. When the blot was stripped and hybridized with a probe specific for the 5' HER-2 coding sequence, a broad band representing the 4.8 and 4.5 kb mRNAs was detected in fetal kidney tissues and the truncated 2.6 kb transcript was detected in liver showing that these alternative transcripts contain sequences that encode the

HER-2 ECD. Because the inserted ECDIIIa sequence contains a termination codon, both of these alternative transcripts likely encode the same truncated protein product. These findings show that two alternative transcripts containing the ECDIIIa sequence are expressed in a tissue-specific manner in normal human tissues, and that the 4.8 kb alternative transcript is expressed in the HEK-293 cell line.

Expression of a secreted protein of ~68 kDa containing the ECDIIIa sequence To assess whether the alternative sequence is translated into a protein product, we expressed the ECDIIIa sequence as a polyhistidine-tagged peptide in bacteria, purified the peptide by nickel-affinity chromatography, and raised antisera against the purified peptide. The HEK-293 cells, which expressed the 4.8 kb ECDIIIa alternative transcript, were examined for expression of an ECDIIIa-containing protein by Western analysis. A 68 kDa protein from the cell extract and from the extracellular media reacted with the anti-ECDIIIa antibody (Fig. 3) but not with preimmune sera (data not shown) and reactivity was blocked by preincubation of the antisera with purified ECDIIIa peptide (Fig. 3). The 68kDa protein was further characterized as the product of the alternative transcript based on its reactivity with antipeptide antibody against residues 151-165 of p185HER-2 (Lin & Clinton, 1991). The larger protein of ~125 kDa detected in some cases may be an aggregate of p68. The cDNA sequence of the alternative transcript (Fig.1) predicts a secreted protein product of 65-70 kDa if all five consensus N-linked glycosylation sites in the N-terminal p185HER-2 sequence are glycosylated (Stern et al., 1986).

The expression of p68 relative to p185HER-2 is markedly reduced in carcinoma cell lines in which the HER-2 gene is amplified. Although the alternative transcript containing the ECDIIIa sequence was detected in human fetal tissues and in HEK-293 cells, it could not be detected by Northern analysis of SKBR-3, BT474, and SKOV-3 carcinoma cell lines, which all have their HER-2 gene amplified about 8 times (Kraus et al., 1987). Therefore the more sensitive ribonuclease protection assay (RPA) was employed using an antisense probe which spanned the entire ECDIIIa sequence and 5' HER-2 exon sequence flanking

the ECDIIIa sequence. The alternative HER-2 mRNA with the ECDIIIa insert was detected at less than 5% of the fully spliced transcript in SKOV-3, SKBR-3, and BT474 cells, and was expressed at nearly equivalent levels to the p185HER-2 transcript in HEK-293 cells (Fig. 4). Therefore, the carcinoma cells with HER-2 gene amplification express relatively reduced amounts of the alternative transcript at less than 5% of the 4.5 kb HER-2 transcript.

We next examined the relative proportions of p68 and p185HER-2 proteins in several cell lines with and without HER-2 gene amplification. Western blots were prepared and probed with both antisera specific for p68 and for p185HER-2. Figure 5 shows that p185 was readily detected in the carcinoma cells lines that have their HER-2 gene amplified. However, there was not a corresponding elevation in p68. In comparison, p68 was the only HER-2 protein detected in the HEK-293, IOSEVAN, and HBL100 nontumorigenic cells, although p185 is expressed at very low levels in these cells. The relative levels of cellular p68 were also reflected in the amount secreted from each of these cells (data not shown). This result shows that the p68 protein and mRNA are low in proportion to that of p185 in cells with HER-2 gene amplification and indicates that a mechanism may exist to maintain low levels of p68 when HER-2 is amplified in carcinoma cells.

The ECDIIIa protein specifically binds to p185HER-2 at nM affinity. Because the ECDIIIa protein is secreted and contains a novel proline-rich C-terminus, we next examined its binding properties. The His-tagged protein expressed from the p68ECDIIIa cDNA as a 50 kDa unglycosylated protein in bacteria was purified and radiolabelled with 125I. Increasing concentrations of radiolabelled p50 ECDIIIa protein were added to HER-2 transfected 17-3-1 cells and to the parental 3T3 cells. Saturation binding to the transfected 17-3-1 cells which overexpress p185HER-2 was observed, while binding to the parental 3T3 cells was not above background levels (Fig. 6A). Scatchard analysis suggested a single, high affinity site and predicted an apparent dissociation constant of about 14 nM. To test whether the ligand binds specifically to p185HER-2, the radiolabelled p50ECDIIIa was chemically cross-linked to intact 17-3-1 cells, which were washed, extracted, and immunoprecipitated with antibodies specific for p185HER-2.

Figure 6B shows that p50ECDIIIa was specifically cross-linked to p185. It was therefore concluded that the ECDIIIa protein is a ligand for p185HER-2.

The anchorage-independent growth of cells that overexpress p185HER-2 is inhibited in the presence of the ECDIIIa ligand. Because SKOV-3 carcinoma cells have very low levels of p68ECDIIIa relative to the p185HER-2 expression, we examined whether increasing p68 levels in the extracellular media affects their anchorage-independent growth. SKOV-3 cells were plated in soft agar cultures in media conditioned by HEK-293 cells (HEK-CM), which contains relatively high levels of p68ECDIIIa. Media conditioned by SKOV-3 cells (SKOV-CM) was used as a control since it does not contain detectable p68 (see Fig. 7, panel B). For the first 4-5 days, colony growth was indistinguishable in the presence (HEK-CM) or absence of p68 (SKOV-CM). However, at 5-7 days the cultures that contained p68ECDIIIa stopped growing and appeared to undergo apoptosis. This same pattern of initial growth and then death was reproducibly observed in four separate experiments using different preparations of p68ECDIIIa. The number of colonies, consisting of 50 cells or more, was reduced several fold in the presence of p68 (HEK-CM) compared to control cultures without CM or with SKOV-CM (Fig.7 A ,B). The 17-3-1 cell line, like SKOV-3 cells (Deshane et al., 1995; Juhl et al., 1997), depends on p185 overexpression for anchorage-independent growth (DiFiore et al., 1987; Hudziak et al., 1987). 17-3-1 colony formation was also inhibited in the presence of p68ECDIIIa in a manner similar to that observed for SKOV-3 cells (Fig. 7 A, B). In contrast, the NIH-3T3 cells transformed by the *src* oncogene (*src* 527) grew normally in the presence of p68ECDIIIa indicating that the inhibitory effect was specific for cells transformed by p185HER-2 (Fig 7 A,B). Further evidence that the ECDIIIa ligand inhibits p185-mediated anchorage-independent growth was gained by using the His-tagged ECDIIIa protein, p50ECDIIIa, purified from bacteria. P50ECDIIIa inhibited the anchorage-independent growth of 17-3-1 cells, but not of NIH *src* cells, in a dose dependent fashion (Fig. 7C).

The ECDIIIa ligand inhibits the level of p185HER-2 dimers. Since the ECDIIIa protein is a ligand for p185, we examined whether it may have a specific effect on the dimerization state of p185HER-2, since dimerization is required for RTK activation and subsequent signal transduction (Heldin & Ostman, 1996). Addition of p68ECDIIIa from HEK-CM to 17-3-1 cells for 30 min inhibited the levels of p185-containing dimers (Fig. 8). HER-2 dimers were identified with p185HER-2-specific antibodies as an ~360 kDa complex detected only when cells were treated with the homobifunctional crosslinking reagent, BS3 (Fig. 8). Support that dimer inhibition was caused by the ECDIIIa ligand was provided by the addition of purified, recombinant p50ECDIIIa at 200 nM, which eliminated dimers within 10 minutes. The repression of HER-2 dimers by the ECDIIIa ligand appeared to be transient with partial restoration of dimer levels by 2 hrs and return to nearly control levels by 4 hrs. The restoration of dimers in the presence of excess amounts of ECDIIIa ligand could be due to inactivation of the ligand with time in serum-free media, or the ligand may be internalized and degraded. These results suggest that the ECDIIIa ligand causes a rapid loss of HER-2 dimers from17-3-1 cells.

DISCUSSION

An alternative HER-2 transcript encodes a novel secreted protein, p68ECDIIIa. The results presented here demonstrate expression of alternative HER-2 mRNA, which contains an additional 274 nucleotides, probably intron 8. Consistent with this finding, an alternative transcript of ~ 4.8 kb was detected in human fetal kidney tissue and in the human embryonic kidney cell line, HEK 293, and a transcript of 2.6 kb, which is the size expected if the sequence is retained in the 2.3 kb truncated HER-2 mRNA (Yamamoto et al., 1986; Scott et al., 1993), was detected in human fetal liver tissue by Northern blot analysis using a probe specific for the inserted sequence or for the HER-2 ECD coding sequence (Fig. 2). The inserted sequence introduces a termination codon and predicts a novel 79 amino acid extension designated ECDIIIa at residue 340 of the p185 HER-2 protein. The predicted protein therefore lacks the transmembrane

and intracellular domains, but contains subdomains I and II of the extracellular domain of p185. A secreted protein which reacted with antibody against the novel C-terminal sequence was detected (Fig. 3). The ECDIIIa protein was found to be 68 kDa which is the approximate size expected of the protein encoded by the alternative transcript if the five N-linked glycosylation sites found in subdomains I and II of p185 are glycosylated (Stern et al., 1986), while the unglycosylated recombinant protein expressed from the ECDIIIa cDNA in bacteria migrated as a 50 kDa protein.

The ECDIIIa protein is a ligand for p185HER-2. The ECDIIIa protein binds specifically to p185HER-2 at nM affinity. Scatchard analysis indicates a single class of saturable binding sites on HER-2 transfected 17-3-1 cells with a KD of ~14 nM (Fig. 6) An extensive search for soluble secreted factors that specifically activate p185HER-2 has been conducted because of its homology with the EGF-receptor suggesting that p185HER-2 might be a receptor for a growth factor ligand. Although several EGF-like ligands have been discovered, none yet characterized binds directly with high affinity to p185HER-2 (Hynes & Stern, 1994; Dougall et al., 1994; Tzahar & Yarden, 1998). While it is possible that the ECDIIIa ligand described here binds indirectly to p185 through a coreceptor, this seems unlikely since radiolabeled p50ECDIIIa protein was cross-linked to p185 HER-2 at the cell surface (Fig. 6). The high affinity binding of the ECDIIIa protein to p185 appears to be conferred by the novel, intron-encoded ECDIIIa domain, rather than by its N-terminus (Doherty et al., manuscript in preparation), which is identical to subdomains I and II of p185 . HER-2 ECD which contains subdomains I and II, does not detectably bind to p185HER-2 (Tzahar et al., 1997; O'Rourke et al., 1997; Fitzpatrick et al., 1998). P68 differs from previously described EGF family ligands (Groenen et al., 1994) in several respects. P68 lacks an EGF homology domain and it contains the first 340 amino acids of the receptor itself, p185HER-2.

Evidence that p68ECDIIIa is an antagonist of p185HER-2. Binding of all naturally occurring or engineered ligands for mammalian EGFR family members, is tightly coupled to stimulation of receptor dimerization and tyrosine phosphorylation (Hynes & Stern, 1994;

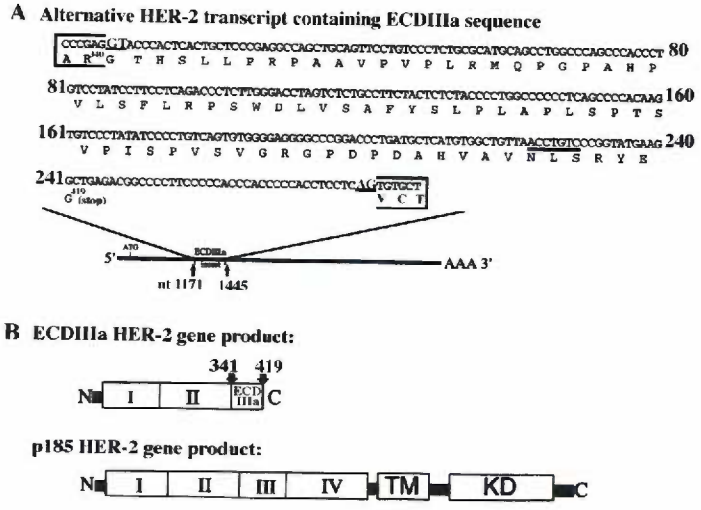
Dougall et al., 1994; Tzahar & Yarden, 1998; Groenen et al., 1994). Although p68ECDIIIa binds with high affinity to p185, it did not stimulate receptor tyrosine phosphorylation (data not shown). Rather, this novel ligand was found to down-regulate dimers of p185 (Fig. 8) suggesting it acts as an antagonist. The addition of ECDIIIa ligand, produced in mammalian cells (HEK-CM) or purified from bacteria, to 17-3-1 cells rapidly reduced dimers to undetectable levels (Fig. 8). Disappearance of dimers within 10 minutes suggests that ECDIIIa either disrupts existing dimers, shifts the equilibrium between dimers and monomers by stabilizing monomers, or causes down-regulation and degradation of dimers. The ECDIIIa ligand may inhibit dimerization and subsequent receptor activation in a dominant negative fashion by occupying monomeric receptors and blocking their recruitment into dimers (O'Rourke et al., 1997). It is also possible that the ECDIIIa ligand initiates a novel HER-2 signaling pathway by stimulating endocytosis and down-regulation of the ligand/receptor complex. Few natural antagonists for RTKs have been identified to date. The Argos protein, is an example of an extracellular inhibitor of the Drosophila EGF receptor (Schweitzer et al., 1995). Likewise, Angiopoietin-2 is a natural antagonist for the Tie 2 endothelial RTK (Maisonpierre et al., 1997).

If p68ECDIIIa interferes with dimerization, then down-stream events, including oncogenic signaling by p185, may be obstructed by p68 (Tzahar & Yarden, 1998). Colony formation in soft-agar by two cell lines that both require p185 overexpression for their tumorigenic growth, SKOV-3 (Deshane et al., 1995; Juhl et al., 1997) and 17-3-1 cells (Lonardo et al., 1990; DiFiore et al., 1987), was inhibited in the presence of p68ECDIIIa. Anchorage-independent growth of colonies in soft agar is an *in vitro* assay, which reflects the tumorigenic properties of HER-2 transformed cells (DiFiore et al., 1987; Hudziak et al., 1987). The repressive effect of the ECDIIIa ligand appears to be specific for HER-2 transformed cells since NIH-3T3 cells transformed by *src*527 were not inhibited in the presence of the ECDIIIa ligand (Fig. 7). Growth inhibition by the ECDIIIa protein could provide a selective pressure for overexpression of p185 relative to p68ECDIIIa in tumor cells with HER-2 gene amplification (Figs. 4 & 5).

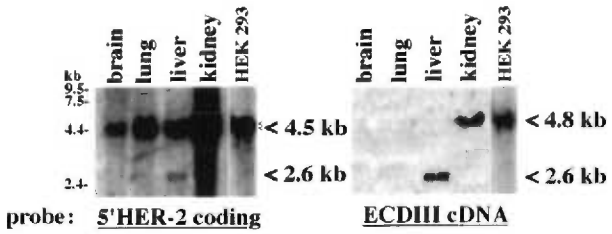
In summary, our results support the model that p68ECDIIIa is a naturally occurring antagonistic ligand for p185HER-2, which we have named Herstatin. Since p185HER-2 appears to be exceptionally active and interactive with other EGFR family members (Tzahar & Yarden, 1998), Herstatin may provide a mechanism for dampening that activity in a tissue-specific fashion (Fig. 2). Moreover, as a growth inhibitor (Fig. 7), Herstatin could have therapeutic value against human cancers that are driven by overexpression of p185HER-2.

Chapter 2 Figures

ch. 2, Fig. 1. Nucleotide sequence of the 274 nucleotide insertion and deduced amino acid sequence. The HER-2 ECD coding sequence from exons 1-9 was amplified by PCR from a cDNA library from SKOV-3 cells. A product of ~1420 bp, found to be HER-2-specific by Southern blot analysis, was subcloned and the nucleotide sequence was determined. **A** The nucleotide sequence is shown for the 274 bp insert (outside the box) and for the immediately adjacent 5' and 3' sequences enclosed in the box. The insertion is located between nucleotide residues 1171 and 1172 and following amino acid residue 340 in p185HER-2 using the numbering of Coussens et al. (1985). The consensus 5' and 3' splice sites are underlined and shown in larger print. The inserted sequence is in-frame with 5' HER-2 exon sequence and is deduced to encode a 79 amino acid extension following Arg 340 (R340). The novel 79 amino acid sequence encoded by the insert is proline-rich (19%) and has a consensus asparagine linked glycosylation site, which is underlined. A stop codon (stop) is found within the inserted sequence. **B** The predicted product of the alternative transcript is a truncated secreted protein which contains subdomains I and II identical to p185 and is missing the transmembrane domain and cytoplasmic domain. If fully glycosylated, the expected size is 65-70 kDa. For comparison, the schematic structure of p185 HER-2 indicates subdomains I, II, III, and IV in the ECD, the transmembrane domain (TM), and the kinase domain (KD).


A Alternative HER-2 transcript containing ECDIIIa sequence
80
81
160
161
240
241
G (stop)
V C T
ATG
ECDIIIa
5'
AAA 3'
nt 1171
1445
B ECDIIIa HER-2 gene product:
341
419
N
I
II
ECD IIIa
C
p185 HER-2 gene product:
N
I
II
III
IV
TM
KD
C

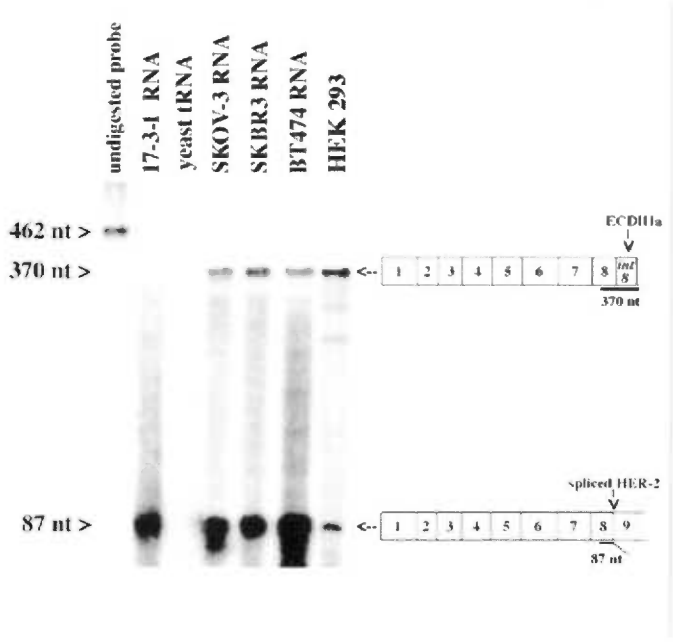
ch. 2, Fig. 2. Detection of alternative HER-2 transcripts containing the ECDIIIa sequence by Northern blot analysis. PolyA+ mRNA (2.5 μg) from different human fetal tissues (Clontech) or isolated from HEK-293 cells was subjected to Northern blot analysis using a ^{32}P-labeled antisense RNA probe complimentary to the ECDIIIa sequence. The blot was stripped and reprobed with a ^{32}P-labeled cDNA probe specific for the 5' HER-2 exon sequence.


brain
lung
liver
kidney
HEK 293
4.4-
2.4-
< 4.5 kb
< 2.6 kb
brain
lung
liver
kidney
HEK 293
< 4.8 kb
< 2.6 kb
probe: 5'HER-2 coding
ECDIII cDNA

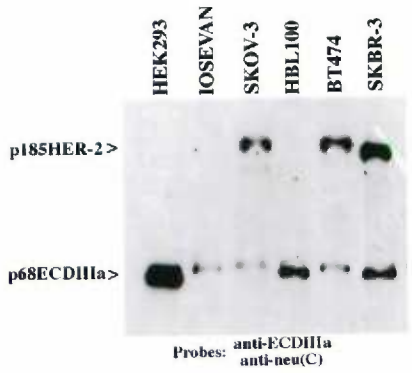
ch. 2, Fig. 3. Sequence-specific reactivity of anti-ECDIIIa domain antibody with a protein of ~ 68 kDa in HEK-293 cells. Cell extract protein (20 μg) and 20 μl of media conditioned by HEK-293 cells, as described in Methods, were Western blotted and probed with anti-ECDIIIa diluted 1:10,000 (lanes 1 and 2) or with anti-ECDIIIA diluted 1:10,000 containing 50 μg/ml purified His-tagged ECDIIIa peptide (lanes 3, 4).


cellular
extracellular
cellular
extracellular
p68ECDIIIa
1
2
3
4

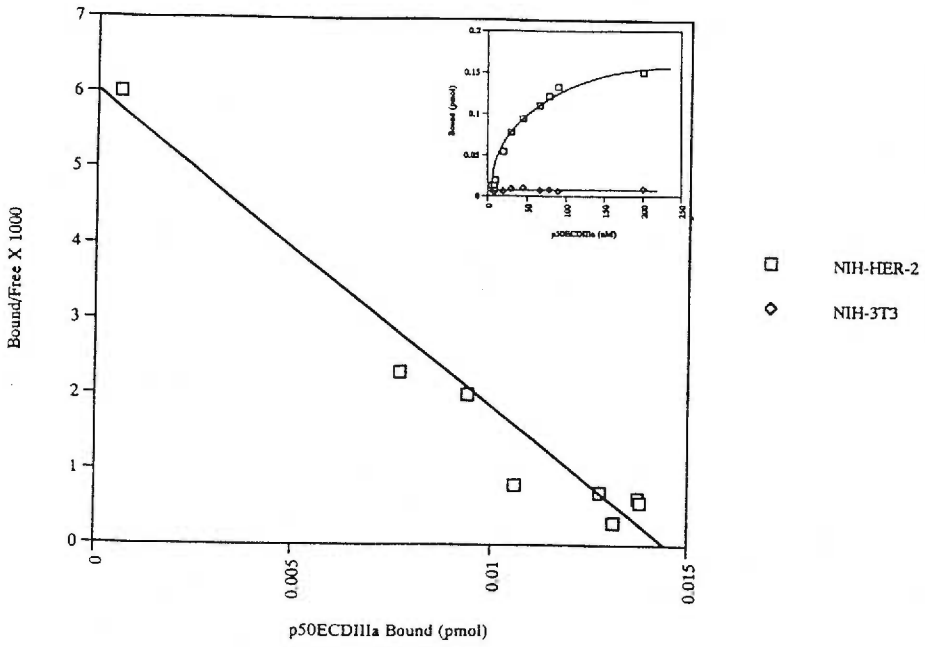
ch. 2, Fig. 4. Ribonuclease protection assay to detect p68ECDIIIa mRNA and p185HER-2 mRNA. A ^{32}P-labeled antisense RNA probe, complimentary to the entire ECDIIIa sequence and to 87 nt of 5' flanking exon sequence was hybridized to RNA from the different cell lines. The hybrids were digested and analyzed as described in Methods. A protected fragment of 370 nt which corresponds to the size expected for RNA containing 5' HER-2 exon sequence and ECDIIIa sequence is illustrated schematically on the right and was detected in SKOV-3, SKBR-3, and BT474 RNA, but not in yeast tRNA nor in 17-3-1 cells which are transfected with HER-2 cDNA. A protected fragment of 87 nt, which corresponds to the size expected for RNA containing 5' exon sequence but not ECDIIIa sequence, illustrated on the right, was detected in all cell lines but not in tRNA.

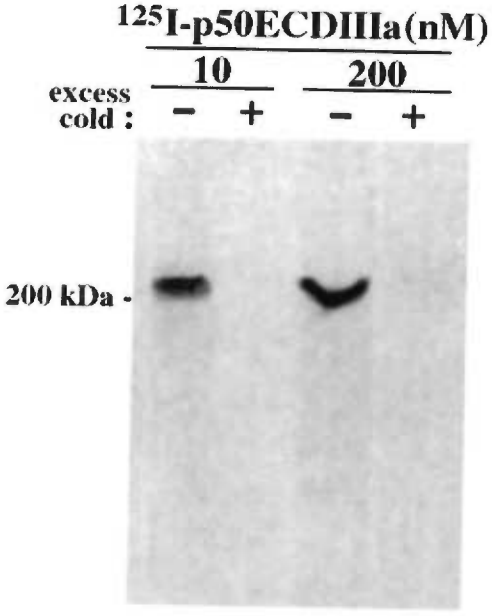

undigested probe
17-3-1 RNA
yeast tRNA
SKOV-3 RNA
SKBR3 RNA
BT474 RNA
HEK 293
462 nt >
370 nt >
87 nt >
ECDHIa
int 8
370 nt
spliced HER-2
87 nt

ch. 2, Fig. 5. The expression of p185HER-2 relative to p68ECDIIIa is markedly elevated in carcinoma cell lines in which the HER-2 gene is amplified. Cell extracts (15 μg of protein) from HEK-293 cells, nontumorigenic ovarian surface epithelial cell line (IOSEVAN), ovarian carcinoma cell line with HER-2 gene amplification (SKOV-3), nontumorigenic breast epithelial cell line (HBL100), and breast carcinoma cell lines with HER-2 gene amplification (BT474 and SKBR-3), were resolved by SDS-PAGE in 7.5% acrylamide gels and analyzed as a Western blot with both antibodies specific for p68 (anti-ECDIIIa) and for p185 (anti-neu(C)).

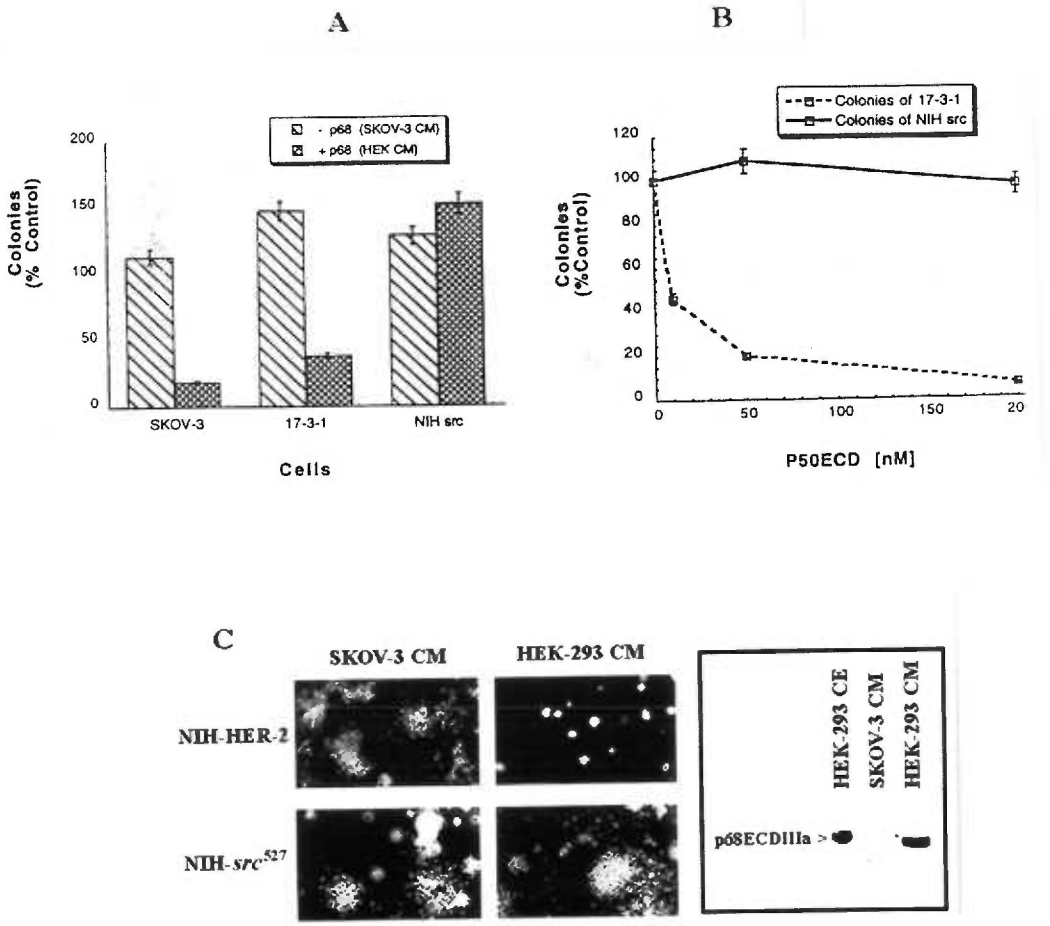

HEK293
IOSEVAN
SKOV-3
HBL100
BT474
SKBR-3
p185HER-2>
p68ECDIIIa>
Probes: anti-ECDIIIa
anti-neu(C)

ch. 2, Fig. 6. The ECDIIIa protein specifically binds at nM affinity to p185HER-2. **A** Various concentrations of radiolabeled p50ECDIIIa were incubated with HER-2 transfected 17-3-1 cells or untransfected 3T3 cells. Non-specific binding was determined in the presence of a 100-fold excess of the unlabeled p50 and was subtracted from the total amount of bound radioactivity. Binding results were analyzed by using the Scatchard method and by plotting the saturation curve (inset). **B** Radiolabelled p50, in the presence and absence of 100-fold excess unlabeled p50, was bound to 17-3-1 cells and then incubated with the crosslinking reagent, BS^3. The washed cells were extracted and immunoprecipitated with 5 μl of anti-neu(C) as described (23). The immune complex was washed, resolved by SDS-PAGE, and radiolabelled p50 was detected by autoradiography.

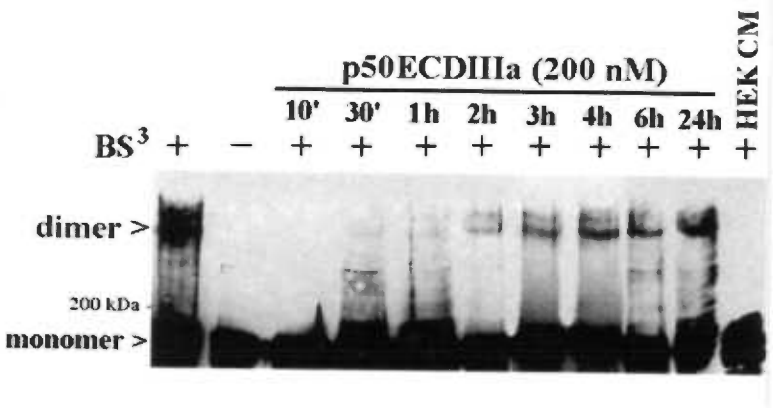

Bound/Free X 1000
p50ECDIIIa Bound (pmol)
0
1
2
3
4
5
6
7
0
0.005
0.01
0.015
NIH-HER-2
NIH-3T3


125I-p50ECDIIIa(nM)
10
200
excess cold :
- + - +
200 kDa -

ch. 2, Fig. 7. Anchorage-independent growth of cells in the presence and absence of the ECDIIIa ligand. Cells were grown as colonies in soft-agar as described in Methods. Colonies of 50 or more cells were counted in triplicate wells and expressed as mean percentages of untreated controls. In panels **A** and **B**, Colony formation was in the absence or presence of conditioned media (200 μg protein) prepared from SKOV-3 cells (SKOV-CM) or from HEK-293 cells (HEK-CM). Panel **B** illustrates the morphology of the colonies grown in HEK-CM or SKOV-CM. On the right is the Western analysis showing the p68ECDIIIa levels in10 μg of protein from SKOV-3 CM, HEK-293 CM, or from HEK-293 cell extracts. We estimate that there is 20 nM p68ECDIIIa in HEK-CM concentrated 100-fold based on comparisons with the Western signal using known amounts of purified ECDIIIa protein. In panel **C** P50ECDIIIa, purified from bacteria, was added at the indicated concentrations to the soft agar cultures. The ligand vehicle containing 50mM Tris pH 9.5 and 1mM DTT was added at the same concentration to all cultures.


A
B
- p68 (SKOV-3 CM)
+ p68 (HEK CM)
Colonies (% Control)
200
150
100
50
0
SKOV-3
17-3-1
NIH src
Cells
Colonies of 17-3-1
Colonies of NIH src
Colonies (%Control)
120
100
80
60
40
20
0
0
50
100
150
20
P50ECD [nM]
C
SKOV-3 CM
HEK-293 CM
NIH-HER-2
NIH-src527
HEK-293 CE
SKOV-3 CM
HEK-293 CM
p68ECDIIIa >

ch. 2, Fig. 8. The ECDIIIa ligand down-regulates HER-2 dimers. 17-3-1 cells were incubated at 37° C for the indicated times in binding buffer with or without 200 nM p50ECDIIIa from bacteria or 100 μg protein from HEK-CM. Cells were incubated with the crosslinking reagent BS^3, extracted, and resolved in 5% polyacrylamide gels, which were analyzed as a Western blot with anti-neu(C).



Chapter 3

An Alternative HER-2/*neu* Transcript of 8 kb has an Extended 3'UTR and Displays Increased Stability in SKOV-3 Ovarian Carcinoma Cells

A Manuscript submitted to the Journal *Gynecologic Oncology*

by

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ABSTRACT

HER-2/*neu* is a potent oncogene that predicts poor outcome when overexpressed in ovarian cancer. The SKOV-3 ovarian carcinoma cell line, one of the only models for HER2-driven ovarian cancer, expresses a major uncharacterized 8 kb alternative HER-2 transcript. The aim of this study was to characterize the structure, determine the origin of the alternative sequence, and examine the possible role of the 8 kb alternative transcript in overexpression of the HER-2 gene. The structure of the 8 kb transcript was investigated using the polymerase chain reaction (PCR) and nucleotide sequencing of cDNA clones. PCR analysis of genomic DNA was used to assess the origin of the 8 kb transcript. The stability of the 8 kb mRNA was assessed by Northern blot analysis of RNA extracted from cells treated with transcriptional inhibitors. Similar 5'UTR and coding sequence but an extended 3'UTR was contained in the 8 kb compared to the well-characterized 4.5 kb HER-2 transcript. Genomic DNA had continuity between the novel 3'UTR sequence from the 8 kb transcript and adjacent HER-2 terminal exon sequence. The 8 kb transcript had a half-life of 13 h compared to 5.5 h for the 4.5 kb transcript ($p<0.01$). The 8 kb transcript is generated from alternative polyadenylation site usage rather than gene rearrangement. Since the 8 kb transcript contains alternative sequence found at the 3'end of the normal HER-2 gene, it could be expressed in other cells. Increased stability of the 8 kb transcript may confer a selective advantage for SKOV-3 cells by providing enhanced HER-2 expression.

INTRODUCTION

The HER-2/*neu* (c-erbB-2) oncogene is expressed in several human tissues as a 185 kDa transmembrane tyrosine kinase (p185HER-2) with extensive homology to the Epidermal Growth Factor Receptor (EGFR) (Coussens et al., 1985; Yamamoto et al., 1986). p185HER-2 signals through the mitogen-activated protein kinase pathway, inducing nuclear events that lead to proliferation of tumor cells (Ben-Levy et al., 1994). Overexpression of HER-2 has been reported in an array of human carcinomas, most notably in up to 30% of breast and ovarian cancers (Hynes & Stern, 1994). Moreover, HER-2 overexpression is associated with more aggressive disease and predicts poor prognosis for survival of the breast and ovarian cancer patient (Slamon et al., 1989).

Evidence suggests that overexpression of the unaltered HER-2 gene is the major aberration of this oncogene in human malignancies (Hynes & Stern, 1994; Slamon et al., 1989, 1987). Indeed, overexpression of the "wildtype" p185HER-2 protein has been shown to cause transformation and malignant tumorigenesis (Rodriguez & Park, 1994; Guy et al., 1992; Segatto et al., 1988; Hudziak et al., 1987; DiFiore et al., 1987). Moreover, depression of p185HER-2 expression by a variety of molecular mechanisms causes tumor regression in animal models (Juhl et al., 1997; Deshane et al., 1995; Weichen et al., 1995; Yu et al., 1995, 1993).

Although HER-2 gene amplification has been shown as one mechanism underlying p185HER-2 overexpression (Slamon et al., 1989; Hung et al., 1992; Tyson et al., 1991), many tumors and cell lines overexpress HER-2 mRNA and protein, in the absence of, or exceeding the level of, gene amplification (Hynes & Stern, 1994; Slamon et al., 1989; Tyson et al., 1991; Hollywod & Hurst, 1993).

A human ovarian carcinoma cell line, SKOV-3, exhibits 8-fold amplification of the HER-2 gene (Karlan et al., 1994; Lichtenstein et al., 1990) and overexpresses both HER-2 mRNA and p185HER-2 by greater than 140-fold relative to most normal cells. Despite remarkably high levels of p185HER-2 in SKOV-3 cells, expression of even more p185HER-2 at the cell surface may further promote malignant growth. This was suggested by the increased p185HER-2 in SKOV-3

cells, selected, from early intraperitoneal metastases in nude mice that displayed stable enhanced metastatic potential (Yu et al., 1993). In addition to increased metastatic potential, the increased p185HER-2 expression correlated with enhanced growth rate of SKOV-3 cells. The additional increase in p185 occurred without evidence of further HER-2/neu gene amplification, suggesting transcriptional or post-transcriptional mechanisms (Yu et al., 1993). Indeed, HER-2 transcription is upregulated in the breast carcinoma cell lines BT474, SKBR3, and ZR75-1 (Hollywood & Hurst, 1993) and post-transcriptional mechanisms for increased HER-2 mRNA and protein expression have been found in HC11 (Kornilova et al., 1992) and in SKOV-3 cells (Karlan et al., 1994; Jones et al., 1994). Therefore, these studies suggest that, in addition to gene amplification, post-transcriptional mechanisms are important for upregulation of HER-2 mRNA levels in carcinoma cells.

In addition to the well-characterized 4.5 kb transcript, alternative HER-2 transcripts of unknown significance have been identified in both human tumors and tumor cell lines (Hung et al., 1992; Karlan et al., 1994; Lichtenstein et al., 1990; Jones et al., 1994; Scott et al., 1993; Fujimoto et al., 1995). SKOV-3 cells, which produce the highest level of p185HER-2 of any carcinoma cell line and are frequently used as a model for studies of HER-2 overexpression, also express an abundant mRNA species of approximately 8 kb (Karlan et al., 1994; Lichtenstein et al., 1990; Jones et al., 1994). While the mechanism underlying generation of this alternative transcript has not been determined, based on the presence of a polymorphism in the HER-2 gene in SKOV-3 cells, it has been suggested that the 8 kb transcript results from a gene rearrangement (Hung et al., 1992).

In this study, we present evidence that the alternative HER-2 transcript contains similar 5'UTR and coding sequences to those of the 4.5 kb transcript. However, it differs in the length of its 3'UTR, which extends by approximately 3.5 kb from the 3'UTR found in the 4.5 kb transcript. Additionally, the 8 kb transcript displays increased stability, which could account for its

overexpression. Our data support the possibility that the alternative 8 kb HER-2/*neu* transcript could confer increased p185HER-2 expression in SKOV-3 cells.

MATERIALS and METHODS

Cell culture. Human breast and ovarian carcinoma cell lines were obtained from American Type Culture Collection (Rockville, MD) maintained in either DMEM (SKBR3 and SKOV-3) or RPMI (BT474), supplemented with 10% fetal bovine serum and 0.05% gentamycin, as described (Hua et al., 1995). All media and supplements were obtained from GIBCO BRL (Life Technologies, Gaithersburg, MD). Unless specified, all other chemicals were purchased from Sigma.

RNA extraction. Cells at 80-90% confluence in 15 cm plates were extracted with TriReagent (Molecular Research Center, Inc., Cincinnati, OH), according to the manufacturer's protocol. RNA was resuspended in RNA sample buffer (50% formamide, 1.5% formalin, 0.1% SDS, 1mM EDTA, 10mM Tris pH 7.4) for Northern blotting, or in 10mM Tris-EDTA, pH 8.0, for mRNA fractionation and reverse transcription.

Isolation of the 8 kb HER-2/neu transcript for sequence analysis Poly-A+ RNA was selected from SKOV-3 cells using the Oligotex mRNA kit (Qiagen). 8 kb mRNA was fractionated from the 4.5 kb mRNA by electroelution from gels by a modification of a previously described method (Harty & O'Callaghan, 1991). Briefly, 0.1 mg SKOV-3 mRNA was electrophoresed on denaturing 3% formalin, 0.7 % agarose gels and gel regions containing mRNA of ~8 kb and ~4-5 kb were excised. The mRNA was electroeluted from the gel slices into dialysis tubing and formaldehyde was removed by dialysis against deionized water. The electroeluted mRNA was used as template for reverse transcription.

Polymerase chain reaction Fractionated mRNA was reverse-transcribed into first strand cDNA using GIBCO BRL (Life Technologies) Superscript RTII System with either random hexamers (for 5'RACE) or oligo-dT12-18 (for 3'RACE and for coding region amplifications).

First strand cDNAs were used as template for PCR using HER-2 sequence-specific primers (GIBCO BRL, Life Technologies) and the Expand High Fidelity PCR System (Boerhinger Mannheim) with 2.5 mM MgCl2, 5 mM of each primer, and 200 mM dNTPs. Cycling parameters were: 30 cycles of 94° C for 30"; 58° C for 45"; 68° C for 3'.

For coding region amplification, a forward primer (5'-TGAGCACCATGGAGCTGGC-3') identical to nt 143-161 of HER-2 cDNA and reverse primer (5'-TCACACTGGCACGTCCAGACC-3') complimentary to nt 3898-3918 of HER-2 cDNA were used to amplify a 3.5 kb HER-2 fragment corresponding to the entire coding sequence (Coussens et al., 1985) of HER-2 cDNA (i.e., from start to stop codons). Numbering of nucleotide and amino acid residues is according to Coussens et al. (1985).

For 5'RACE, single-stranded (or first strand) cDNAs synthesized from 8 kb and 4-5 kb fractionated mRNA were tailed with poly-deoxyadenosine using terminal deoxytranferase (Boehringer Mannheim). Oligo-dT12-18 was used to prime from the newly-synthesized poly-dA tail and a HER-2 sequence-specific reverse primer (5'-GGTGCACACTTGGGTGCTCG-3') complimentary to nucleotides 212-231 of HER-2 cDNA (Coussens et al., 1985) were used in the PCR.

For 3'RACE, first strand cDNAs were amplified using a forward primer identical to nt 3881-3898 of HER-2 cDNA (5'-AGAACCCAGAGTACCTGG-3'), and oligo-dT 12-18 as a reverse primer.

For PCR analysis of genomic DNA, the region spanning the polyadenylation signal sequence utilized in the 4.5 kb HER-2 transcript (Coussens et al., 1985) was amplified from SKOV-3, BT474, SKBR3, and normal human genomic DNA (a gift from Dr. Mike Litt, Oregon Health Sciences University), using a forward primer identical to nucleotides 4301-4323 of HER-2 cDNA and a reverse primer complimentary to unique sequence located approximately 100 bp downstream from the putative polyadenylation signal. DNA was prepared according to the

procedure of Strauss (1998) and the PCR was performed for 25 cycles with cycling parameters of 94° C for 30"; 62° C for 30"; 72° C for 60".

Construction of a cDNA library from size-fractionated SKOV-3 mRNA Fractionated 8 kb mRNA was used as a template for cDNA library construction using a lZIPLOX kit with EcoR1 adaptors per manufacturer's protocol (GIBCO BRL, Life Technologies). Phage were packaged at room temperature for 1 h using a lamda phage packaging kit (Stratagene, La Jolla, CA). Complete phage were then used to infect Y1090 competent cells and the library was plated at ~25,000 pfu per 15 cm plate. Plates were incubated at 37° C for 16 h and screened using GeneScreen Plus Hybridization filters (NEN Life Sciences). Nucleic acids, fixed to membranes by UV crosslinking, were prehybridized for 2 h at 42° C in 30% formamide, 5M NaCl, and 1% SDS with 10 mg/ml of herring sperm DNA as blocking agent. A probe consisting of 108 cpm γ-(^{32}P)ATP end-labelled oligonucleotide, which is identical to nucleotides 4258-4276 of HER-2 cDNA (1), was hybridized with filters for 24 h at 42° C. Filters were then washed in several changes of 0.2XSSC at 55° C and exposed to film. DNA was eluted from positive clones and used to infect D12S cells, plated on LB agar containing 30 mg/ml kanamycin, 0.1 mg/ml IPTG and 0.015% X-gal. lZipLox plasmid DNA (pZL) was purified and sequenced in the Vollum Core Sequencing facility (Portland, Oregon) using universal m13 forward and reverse primers.

Northern blotting was performed as described previously (Hua et al., 1995) using 2.5 mg/lane of poly-A+ RNA. Hybridization was with 107 cpm of random primed α(^{32}P)dCTP-labelled probe of either a 570 bp Xho1-EcoR1 fragment (5' coding region-specific) from the HER-2 expression vector p9002 (Applied BioTechnologies) or a 280 bp HincII cDNA fragment of the unique sequence obtained from an SKOV-3 cDNA library clone, synthesized using a Random Prime DNA Labelling Kit (Boehringer Mannheim). Blots were analyzed by phosphorimaging (Molecular Dynamics).

RESULTS

The alternative 8 kb transcript contains sequences from the 4.5 kb transcript. Northern blot hybridization studies revealed expression of an abundant aberrant HER-2 transcript of 8 kb in SKOV-3 cells (Fig.1). This alternative transcript is polyadenylated, since it was more abundant in poly-A+ selected RNA compared to total RNA. To assess the sequence content of the alternative HER-2 transcript, Northern blots were hybridized with probes corresponding to 5' coding, 3' coding and 3'UTR sequences contained in the 4.5 kb transcript. No difference was detected in the hybridization efficiencies of the different probes with the 8 kb and 4.5 kb transcripts, suggesting that the 8 kb transcript contains a single copy of the HER-2 coding sequence.

The coding sequence and the 5'UTR of the 8 kb and 4.5 kb transcripts are the same size. To examine whether the entire HER-2 coding sequence was contained in the 8 kb transcript, PCR amplification of the 3.5 kb open reading frame of the HER-2 cDNA sequence was targeted using forward and reverse primers flanking the translation initiation and termination codons. Amplification by RT-PCR of SKOV-3 mRNA of ~ 8 kb fractionated by size as described in Methods indicated that the 8 kb transcript contained HER-2 coding sequence of the same size (Fig. 2A, left panel), the identity of which was verified by Southern blot analysis (Fig. 2A, right panel). Sequence identity was further verified by restriction digest and sequence analysis of the PCR products (data not shown), indicating similar sequence content with identical sequence near the initiation and termination codons.

The 5'UTR of the 8 kb and 4.5 kb HER-2 transcripts are the same size. To further examine the structure of the 8 kb alternative transcript, we compared the 5'UTR of the 4.5 and 8 kb HER-2 transcripts using RT-PCR and 5' Rapid Amplification of cDNA Ends (5'RACE). HER-2 5'RACE revealed an amplification product of about 250 bp from both the 4.5 kb and 8 kb cDNA fractions (Fig. 2B, left panel), the identity of which was verified by Southern blot

hybridization (Fig. 2B, right panel). This is the size expected for the normal 5'UTR of the 4.5 kb transcript, indicating no differences in the size of the 5'UTR of the 8 kb and 4.5 kb transcripts.

The 3'UTR of the 8 kb HER-2 transcript is extended and contains unique sequence. 3' RACE consistently yielded nonspecific amplification products of heterogeneous sizes generated from the 8 kb mRNA, while a PCR product of the expected size (~650 bp) was easily obtained from the 4.5 kb mRNA fraction and from reverse-transcribed mRNA from other cells (Fig. 2C). Since the same template was used to amplify the coding sequence and 5'UTR of the 8 kb mRNA, the alternative transcript appears to differ in its 3'UTR.

To directly investigate the sequence of the 3'UTR, an SKOV-3 cDNA library was constructed using fractionated SKOV-3 mRNA enriched in transcripts of ~8 kb as starting material. The SKOV-3 cDNA library was screened using a probe for the HER-2 3'UTR. Six of seven clones contained partial coding and 3'UTR sequence of HER-2 cDNA consistent with the reported sequence for the 4.5 kb transcript (Coussens et al., 1985). A single clone of approximately 7 kb was isolated and found to contain HER-2 3'coding sequence and 3'UTR identical to nucleotides 3138 to 4531, including the termination codon at nt 3916-3918. The sequence of this clone diverged 13 nt following the polyadenylation signal, which is reported to be utilized in generation of the 4.5 kb transcript (Coussens et al., 1985). Partial sequence of this clone is shown in Figure 3, indicating the polyadenylation signal (underlined) which is identical to that reported for the 4.5 kb transcript (Coussens et al., 1985), and additional sequence that continues for another 2 kb. The novel sequence contains multiple termination codons in all six reading frames, further indicating that it is untranslated. The extended sequence is unique with no homologous sequence identified in GenBank and Blast (Entrez) database programs. .

To verify that this sequence was specific to the 8 kb HER-2 transcript, a Northern blot of SKOV-3 and T47D mRNA was probed with the unique sequence (Fig. 4B) and with 5' HER-2 coding sequence, as a control (Fig. 4A). A 280 bp HincII fragment of the unique 3'UTR sequence hybridized only with the 8 kb transcript in SKOV-3 but not with mRNA from T47D, a carcinoma

cell line that does not express the 8 kb alternative HER-2 transcript. Therefore, this sequence is uniquely contained in the alternative 8 kb HER-2 transcript expressed in SKOV-3.

The extended 3'UTR of the 8 kb HER-2/neu transcript results from read-through of polyadenylation signals. Southern blot analysis has revealed the presence of an EcoR1 restriction fragment length polymorphism (RFLP) in the HER-2 gene (Fig.5 and refs.: Hung et al., 1992; Karlan et al., 1994), which has led to suggestions that the 8 kb transcript is generated as a result of gene rearrangement in SKOV-3 cells (Hung et al., 1992). To determine whether the alternative HER-2 transcript is a product of gene rearrangement or of alternative splicing, genomic DNA was amplified by PCR from SKOV-3, BT474, SKBR-3, and normal human genomic DNA using primers that span the polyadenylation signal from the 4.5 kb HER-2 3'UTR sequence (Coussens et al., 1985) and the unique 3'sequence obtained from the cDNA clone of the 8 kb transcript. BT474 and SKBR-3 cells have HER-2 gene amplification, yet do not express detectable aberrant HER-2 transcripts nor exhibit evidence of gene rearrangement (Hung et al., 1992; Hollywood & Hurst, 1993; Karlan et al., 1994; Jones et al., 1994). The PCR amplified the same size fragment of 312 bp from normal and from carcinoma cell line DNA (Fig. 6). These results indicate that the alternative 3'UTR sequence is contiguous with HER-2 3' terminal exon sequence in the human genome, demonstrating that the aberrant transcript is a result of alternative RNA processing, due to read-through of one and possibly two consensus polyadenylation signals (see underlined poly-A sites in Fig. 3). Therefore, the 8 kb mRNA is a direct product of the "wild-type" HER-2 oncogene rather than a product of gene rearrangement.

The alternative 8 kb HER-2 transcript displays increased stability in SKOV-3 cells. To investigate possible mechanisms for abundance of the alternative HER-2 transcript in SKOV-3, we measured its decay rate in comparison with the 4.5 kb transcript. Northern blot analysis was conducted on cells in culture treated with a-amanitin, an inhibitor of transcription (Fig. 7A). The data points were quantitated by phosphorimager analysis, averaged and plotted, and extrapolation of the calculated slope of the best fit line was used to estimate half-life ($t_{1/2}$).

These data demonstrate a $t_{1/2}$ for the 4.5 kb HER-2 transcript of ~5.5 h, which is consistent with previously published determinations in SKOV-3, BT474, and SKBR-3 carcinoma cell lines (Karlan et al., 1994; Jones et al., 1994). However, a significantly greater $t_{1/2}$ of approximately 13 h ($p<0.01$) was obtained for the 8 kb HER-2 transcript (Fig. 7B). A similar result was obtained when an additional inhibitor of transcription, actinomycin D, was employed (data not shown).

DISCUSSION

Although HER-2 gene amplification is a common mechanism for overexpression, many human tumors and cell lines display HER-2 mRNA and protein overexpression in excess of the level accountable by gene copy number, or in the absence of gene amplification (Hollywood & Hurst, 1993). The model ovararian carcinoma cell line, SKOV-3, exhibits the highest level of p185HER-2 expression and exhibits additional mechanisms for upregulation in addition to gene amplification (Yu et al., 1993). Furthermore, in addition to excess expression of the 4.5 kb HER-2 transcript, SKOV-3 also express an abundant alternative HER-2 transcript of 8 kb (Karlan et al., 1994; Lichtenstein et al., 1990; Kornilova et al., 1992; Jones et al., 1994).

Studies designed to repress HER-2 expression in SKOV-3 have targeted both HER-2 mRNA and protein, showing consistent downregulation of p185HER-2 (Juhl et al., 1997; Deshane et al., 1995; Weichen et al., 1995; Yu et al., 1993, 1995). Antisense oligodeoxynucleotides that targeting the HER-2 translation initiation sequence, which is shown here to be intact in the 8 kb transcript, effectively downregulate HER-2 mRNA and p185HER-2 by more than 90% in SKOV-3 cells (Weichen et al., 1995). Further, targeting the HER-2 mRNA in SKOV-3 cells with ribozymes also effectively down-regulated HER-2 protein expression (Juhl et al., 1997). Together with results presented here showing that the initiation sequence and overall coding sequence is intact and that the 8kb transcript is, under some conditions, 3-5 fold more abundant than the 4.5 kb transcript, supports the possibility that it may be translated. However, our findings do not directly show that this alternative transcript is translated into p185HER.

Our results further demonstrate that the 8 kb transcript does not result from a translocation event in SKOV-3, as previously suggested (Hung et al., 1992) and is, thus, a transcription product of an unaltered HER-2 gene. Together, the results of 5'RACE and RT-PCR on 8 kb and 4.5 kb mRNA from SKOV-3 cells indicate that the difference lies only in the extension of the 3'UTR in the 8 kb transcript. PCR analysis of normal genomic DNA revealed that the novel extended 3'UTR sequence in the 8 kb transcript is adjacent to the terminal HER-2 exon in the genomic sequence. Therefore, the 8 kb transcript occurs by read-through of one and possibly two consensus polyadenylation signals.

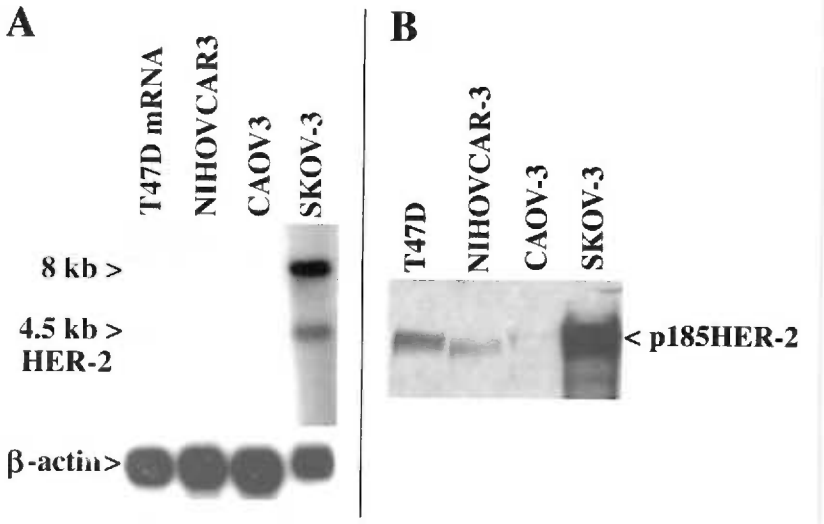
Similarly, an alternative transcript of the EGFR of 10.6 kb has been found in A431 carcinoma cells (Lin et al., 1984) and in human glioblastomas (Sugawa et al., 1990), which both have EGFR gene amplification. This alternative EGFR transcript is reported to result from 3'UTR extension. The significance of this alternative transcript to expression of EGFR has not been characterized.

Significantly, we found that the 8 kb HER-2 transcript displays greater than two-fold increased half-life relative to the 4.5 kb transcript, suggesting that the 8 kb HER-2 is a stable, actively translated transcript, and likely does not contain nonsense mutations, which are known to destabilize mRNA (Beelman & Parker, 1995; Sachs, 1993). The 8 kb transcript had an estimated half-life of 13 hours compared to ~5.5 hours for the 4.5 kb HER-2 transcript, which is consistent with previous reports of approximately 6 hours for the 4.5 kb transcript in SKOV-3 cells (Karlan et al., 1994; Jones et al., 1994). Intrinsically altered HER-2 mRNA stability has not previously been reported. Since the coding region and 5'UTR are similar in the 8 kb and 4.5 kb HER-2 transcripts, it is likely that the extended 3'UTR of the alternative 8 kb HER-2 transcript confers its increased stability. The 3'UTR is known to contain sequences that control cytoplasmic mRNA functions, including stability, translation, and localization (Beelman & Parker, 1995; Jackson & Standart, 1990; Sonnenburg, 1994; Tanguay & Gallie, 1996; Sachs, 1993). Altered mRNA structural stability can result from changes affecting the length of the 3'untranslated region

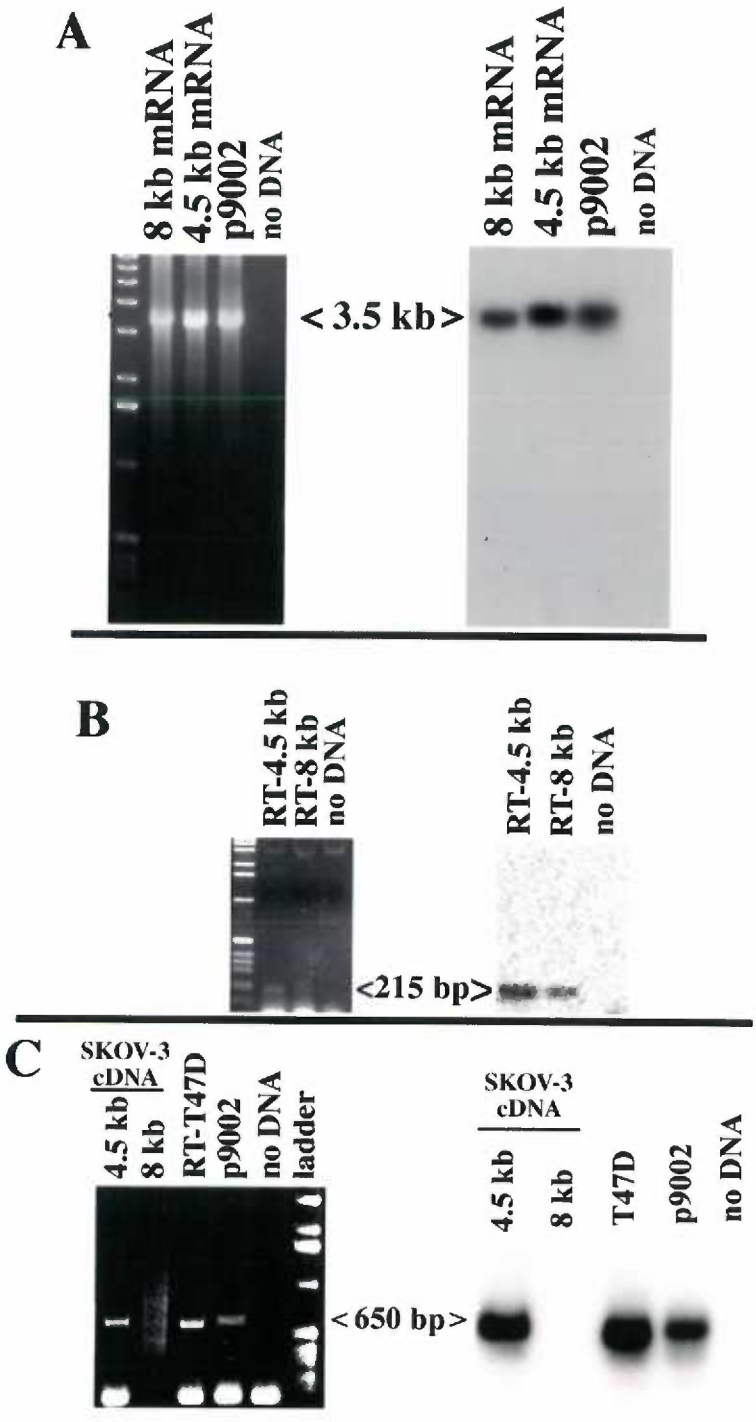
(3'UTR), sequences within the 3'UTR that confer RNA stabilization, including altered stem-loop structures, and/or introduction of binding sites for stabilizing proteins (Beelman & Parker, 1995; Jackson & Standart, 1990; Sonnenburg, 1994; Tanguay & Gallie, 1996; Sachs, 1993). Increased mRNA stability can lead to accumulation of message and, thus, increased translational capacity, resulting in protein overexpression. As observed previously (Yu et al., 1993), an increase in p185HER-2 is associated with a more metastatic phenotype and, thus, expression of the 8 kb message may confer a selective advantage for these aggressive ovarian carcinoma cells in a micrometastatic environment.

Chapter 3 Figures

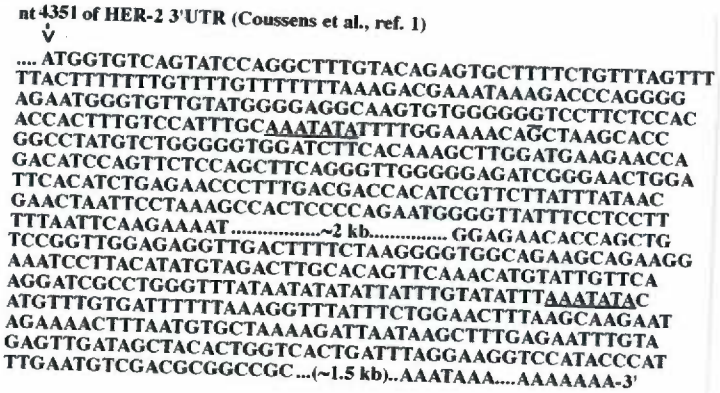
ch. 3, Fig. 1. SKOV-3 cells overexpress a HER-2 transcript of 4.5 kb and 8 kb, and p185HER-2/*neu*. The left panel, **A**, shows a Northern blot of mRNA from T47D, NIHOVCAR3, CAOV3, and SKOV-3 (2.5 μg/lane), hybridized with a 5'HER-2 cDNA probe (top) and a β-actin cDNA probe (bottom) as a loading control. The right panel, **B**, is a western blot of 20 μg protein from each cell line reacted with antibodies against the C-terminus of p185HER-2 (28).


A
T47D mRNA
NHOVCAR3
CAOV3
SKOV-3
8 kb >
4.5 kb >
HER-2
β-actin>
B
T47D
NHOVCAR-3
CAOV-3
SKOV-3
< p185HER-2

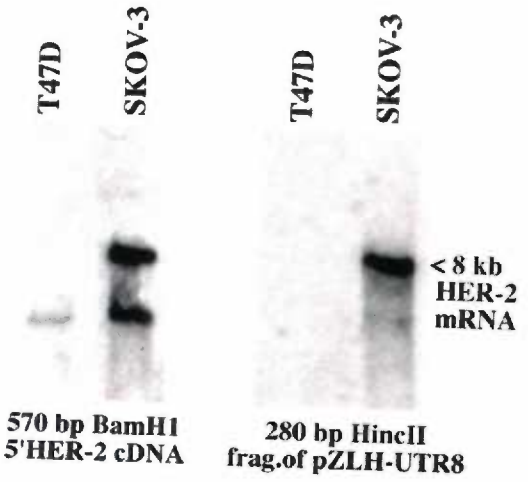
ch. 3, Fig. 2. The 5'UTR and coding sequence of the 8 kb HER-2 transcript are similar to that of the 4.5 kb 'wild-type' transcript, but the 8 kb transcript differs in its 3'UTR. **A:**PCR amplification products from the 8 kb fraction, 4.5 kb fraction, p9002 plasmid containing HER-2 cDNA sequence, and a control without DNA. The right panel is a Southern blot of the gel hybridized with a HER-2 5' coding region-specific probe (described in Methods). **B:** The left panel is an ethidium bromide-stained 2% agarose gel of PCR products from 5' RACE conducted on reverse transcribed SKOV-3 mRNA from the 4.5 kb fraction, the 8kb fraction; and no DNA (control). The right panel is a Southern blot hybridized with a oligonucleotide probe, end-labelled with T4 kinase, which is identical to nucleotides 145-163 of the HER-2 cDNA (1). **C:** The left panel shows 3' RACE products from the 4.5 kb fraction, 8 kb fraction, T47D cDNA, the control plasmid, p9002, and no DNA control. The right panel is a Southern blot of this gel, hybridized with a ^{32}P- labelled HER-2 3'UTR-specific oligonucleotide identical to nt 3918-3943 of HER-2 cDNA.


A
8 kb mRNA
4.5 kb mRNA
p9002
no DNA
< 3.5 kb >
8 kb mRNA
4.5 kb mRNA
p9002
no DNA
B
RT-4.5 kb
RT-8 kb
no DNA
<215 bp>
RT-4.5 kb
RT-8 kb
no DNA
C
SKOV-3 cDNA
4.5 kb
8 kb
RT-T47D
p9002
no DNA
ladder
< 650 bp >
SKOV-3 cDNA
4.5 kb
8 kb
T47D
p9002
no DNA

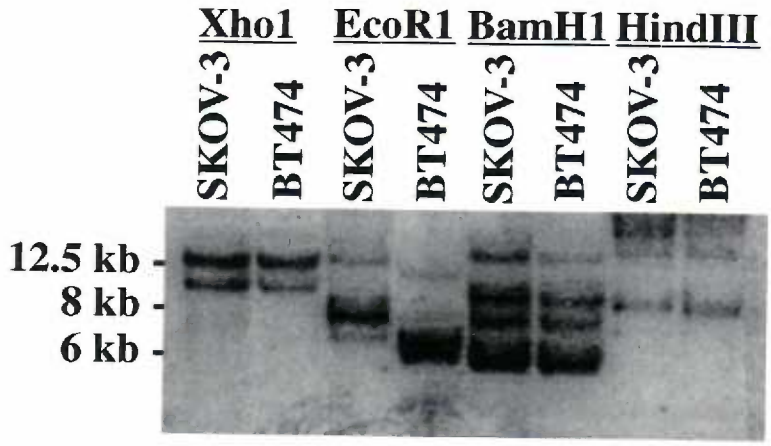
ch.3, Fig. 3. Partial cDNA sequence of an SKOV-3 cDNA library clone containing known HER-2 3'UTR and a unique 3'UTR extension. The putative polyadenylation signal utilized for transcription of the 4.5 kb message is underlined, and, in addition, another consensus polyadenylation signal was found in the unique extended 3'UTR sequence (underlined).


nt 4351 of HER-2 3'UTR (Coussens et al., ref. 1)
.... ATGGTGTCAGTATCCAGGCTTTGTACAGAGTGCTTTTCTGTTTAGTTT
TTACTTTTTTTGTTTTGTTTTTTTAAAGACGAAATAAAGACCCAGGGG
AGAATGGGTGTTGTATGGGGAGGCAAGTGTGGGGGGTCCTTCTCCAC
ACCACTTTGTCCATTTGCAAATATATTTTGGAAAACAGCTAAGCACC
GGCCTATGTCTGGGGGTGGATCTTCACAAAGCTTGGATGAAGAACCA
GACATCCAGTTCTCCAGCTTCAGGGTTGGGGGAGATCGGGAACTGGA
TTCACATCTGAGAACCCTTTGACGACCACATCGTTCTTATTTATAAC
GAACTAATTCCTAAAGCCACTCCCCAGAATGGGGTTATTTCCTCCTT
TTTAATTCAAGAAAAT.....~2 kb..... GGAGAACACCAGCTG
TCCGGTTGGAGAGGTTGACTTTTCTAAGGGGTGGCAGAAGCAGAAGG
AAATCCTTACATATGTAGACTTGCACAGTTCAAACATGTATTGTTCA
AGGATCGCCTGGGTTTATAATATATATTATTTGTATATTTAAATATAC
ATGTTTGTGATTTTTTAAAGGTTTATTTCTGGAACTTTAAGCAAGAAT
AGAAAACTTTAATGTGCTAAAAGATTAATAAGCTTTGAGAATTTGTA
GAGTTGATAGCTACACTGGTCACTGATTTAGGAAGGTCCATACCCAT
TTGAATGTCGACGCGGCCGC...(~1.5 kb)..AAATAAA....AAAAAAA-3'

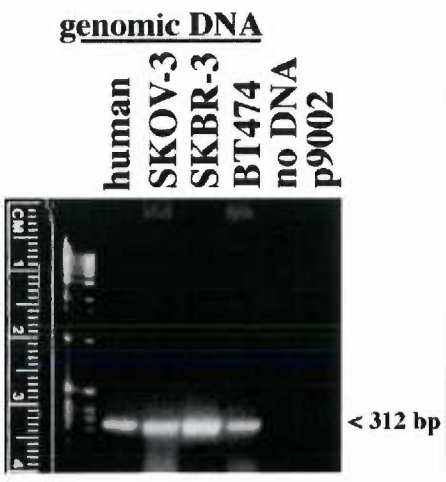
ch. 3, Fig. 4. Sequence-specific hybridization of the novel 3'UTR sequence with 8kb mRNA. Northern blot of T47D (lanes 1, 3) and SKOV-3 (lanes 2, 4) mRNA (2.5 μg/lane), hybridized with 5'HER-2 cDNA (left panel) or unique sequence obtained from the 8 kb cDNA clone (right panel).


T47D
SKOV-3
T47D
SKOV-3
< 8 kb
HER-2
mRNA
570 bp BamH1
5'HER-2 cDNA
280 bp HincII
frag.of pZLH-UTR8

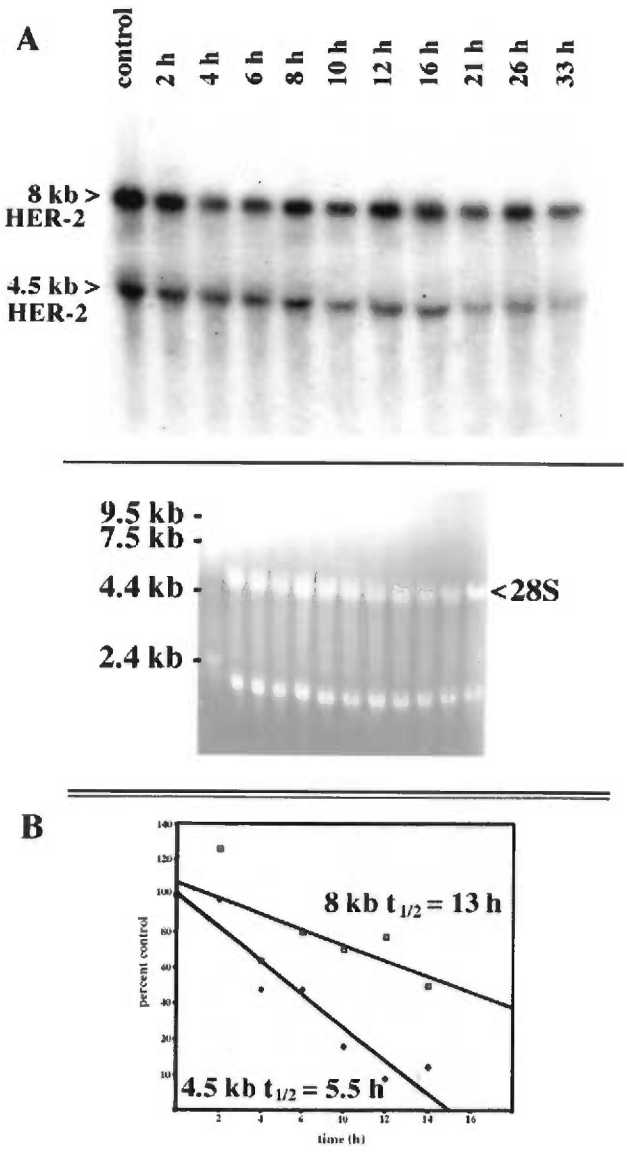
ch. 3, Fig. 5. SKOV-3 cells exhibit EcoR1 restriction fragment length polymorphism in the HER-2 gene. 10 μg of genomic DNA from either SKOV-3 or BT474 cells was digested with excess amounts of restriction enzymes for 24 h at 37° C. Digested DNA was resolved in 0.8% agarose gels, transferred to membrane in 0.4 N NaOH, and hybridized with 10^7 cpm of random-primed ^{32}P-labelled HER-2 cDNA. The blot was washed at high stringency and subjected to phosphorimage analysis.


Xho1
EcoR1
BamH1
HindIII
SKOV-3
BT474
SKOV-3
BT474
SKOV-3
BT474
SKOV-3
BT474
12.5 kb
8 kb
6 kb

ch. 3, Fig. 6. Unique 3'UTR sequence contained within the 8 kb HER-2 transcript is contiguous with HER-2 terminal exon sequence in the genome. Ethidium bromide-stained 1.5% agarose gel of PCR products amplified from genomic DNA of SKOV-3, BT474, IOSEVAN, and normal human blood, or no DNA template. A 312 bp product was amplified from all four DNA templates (lanes 1-4), which is the expected size if the unique 3'UTR sequence from the 8 kb transcript is contiguous with the HER-2 exonic 3'UTR sequence in the genome.


genomic DNA
human
SKOV-3
SKBR-3
BT474
no DNA
p9002
< 312 bp

ch. 3, Fig. 7. The alternative 8kb HER-2 transcript displays increased stability. **A:** The top panel shows a Northern blot of SKOV-3 total RNA (10 µg/lane) extracted at the indicated time points after addition of "-amanitin (24 µg/ml) and hybridized with a HER-2 cDNA probe. The bottom panel shows the 28S RNA in the same gel prior to transfer. **B:** "Best fit line" of data points from five independent experiments. The half-life for each transcript was obtained by extrapolation of 50% of control signal at time "0" or of untreated cells.


A
control
2 h
4 h
6 h
8 h
10 h
12 h
16 h
21 h
26 h
33 h
8 kb > HER-2
4.5 kb > HER-2
9.5 kb -
7.5 kb -
4.4 kb -
2.4 kb -
<28S
B
8 kb t1/2 = 13 h
4.5 kb t1/2 = 5.5 h
percent control
time (h)

Chapter 4

N-terminally Truncated HER-2/neu Protein: Relationship with Shedding of the Extracellular Domain and with Prognostic Factors in Breast Cancer

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by

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(2) The first three authors contributed equally to this work.

ABSTRACT

We identified an N-terminally truncated HER-2/neu product of 95 kDa with *in vitro* kinase activity by western blotting and immunoprecipitations using domain specific antibodies. P95 levels correlated with the extracellular domain (ECD) shed from different cells under varied conditions. Both ECD and p95 were at about 20 fold lower levels in SKOV3 ovarian carcinoma cells compared to BT474 breast carcinoma cells. Both were stimulated by treatment of cells with the phorbol ester tumor promoter (TPA) and the lysosomotrophic agent, chloroquine. The hydroxamate inhibitor of metalloproteases, TAPI, suppressed both p95 and ECD in a dose-dependent fashion with maximal inhibition at 10 μM or less in BT474 cells. Cancer tissues were analyzed by western blotting and scored for p95 and for p185HER-2/neu expression. Breast and ovarian cancer tissues were both found to express p95 in addition to p185HER-2/neu. Of 161 breast cancer tissues, 22.4% expressed p95, 21.7% overexpressed p185, and 14.3% were both p95 positive and overexpressed p185. A higher proportion of node positive patients (23 of 78) than node negative patients (9 of 63) expressed p95 in all tumors combined (P=.032). In the group that overexpressed p185, those that contained p95 were associated with node positive patients (15 of 21) whereas those that were p95 negative were associated with node negative patients (8 of 11) (P=.017). Neither p95 nor p185-rich patients significantly correlated with tumor size or with hormone receptor status in this study. Our findings show that breast cancers, which express the HER-2/neu oncogene, are heterogeneous with respect to HER-2/neu protein products. P95HER-2/neu appears to distinguish tumors that have metastasized to the lymph nodes from those in node negative patients.

INTRODUCTION

The HER-2/neu (erbB-2) gene encodes a receptor-like tyrosine kinase (RTK) which is a member of the epidermal growth factor receptor family (Coussens et al., 1985). Overexpression of HER-2/neu has been observed in tumors arising at many sites including non-small cell lung (Kern et al., 1990), colon (Cohen et al., 1989), prostate (Arai et al., 1997), ovarian, and breast (Slamon et al., 1989). In human breast cancer, where HER-2/neu involvement has been extensively studied, overexpression occurs in 15-30% of the cases (see Singleton & Strickler, 1992) and predicts for significantly lower survival rate and shorter time to relapse in patients with lymph node positive disease (Slamon et al., 1989; Singleton & Strickler, 1992; Slamon et al., 1987; Press et al., 1990; Hynes & Stern, 1994). The significance of HER-2/neu in node negative patients is controversial and so far its clinical utility as a prognostic indicator is limited (Press et al., 1990; Hynes & Stern, 1994). Various approaches are being taken toward HER-2/neu targeted therapeutics many of which are based on antibodies specific to the extracellular domain (ECD) of the transmembrane protein, which either down regulate receptor function or target recombinant toxins with the goal of specifically killing HER-2/neu expressing tumor cells (Press et al., 1990; Hynes & Stern, 1994; Dougall et al., 1994).

In addition to the full length transmembrane product, p185, of the HER-2/neu gene, a truncated product corresponding to the extracellular domain (ECD) is released from breast carcinoma cells in culture by regulated proteolysis (Lin & Clinton, 1991; Zabrecky et al., 1991; Pupa et al., 1993), and is also produced from an alternative transcript (Scott et al., 1993). HER-2/neu ECD is elevated in the serum of patients with breast (Leitzel et al., 1992), ovarian (Maden et al., 1997), and prostate cancer (Myers et al., 1996). Several studies of breast cancers estimate that 6% or less of early stage, about 25% of patients with metastatic and locally advanced disease, and greater than 50% of patients with recurrent metastatic disease have elevated serum ECD (see Brandt-Rauf, 1995). Elevated ECD in serum is associated with overexpression of HER-2/neu in tumor tissue and also reflects tumor load (Molina et al., 1996; Brodowicz, 1997). Soluble HER-

2/neu is a marker of metastatic disease and may predict recurrence (Molina et al., 1996), shortened survival (Brodowicz et al., 1997; Kandl et al., 1994; Fehm et al., 1998), and response to antiestrogen therapy in advanced stage patients (Leitzel et al., 1995; Yamauchi et al., 1997). Serum ECD has also been reported to neutralize the activity of anti HER-2/neu antibodies targeted to the ECD (Baselga et al., 1996; Brodowicz et al., 1997) possibly allowing escape of HER-2-rich tumors from immunological control.

Proteolytic release of the ECD is expected to create an N-terminally truncated, membrane-associated fragment with kinase activity. Cellular fragments created by ectodomain shedding have been described for the colony stimulating factor receptor (CSF-1R) (Downing et al., 1989), the TrkA neurotrophin receptor (Cabrera et al., 1996), Axl receptor (O'Bryan et al., 1995), and HER-4 (Vecchi et al., 1996), while a truncated cellular product of HER-2/neu shedding has not yet been identified. The truncated CSF-1R was found to have *in vitro* kinase activity (Downing et al., 1989), and the cytoplasmic HER-4, induced by phorbol ester tumor promoters, had little or no kinase activity (Vecchi et al., 1997) while a truncated HER-4 found in cells treated with a proteosome inhibitors was an active kinase (Vecchi ety al., 1997).

Several lines of evidence indicate that the ECD of full length transmembrane receptors exerts a negative regulatory constraint on their signaling activity. Engineered deletion of a region of the HER-2 ECD was found to enhance its oncogenic potency (DiFiore et al., 1987). This has also been illustrated by engineered removal of the ECD from the epidermal growth factor (EGF) receptor and by the oncogenic potency of viral encoded v-erbB, v-kit, and v-ros, that are missing regions of the ECD found in their normal cellular counterparts (Rodriguez & Park, 1994). Naturally occurring mutant EGF receptors with N-terminal truncations have been identified in several human carcinomas (Moscatello et al., 1995a) and have constitutive signaling activity and enhanced oncogenic transforming activity in cell culture and animal models (Moscatello et al., 1995b; Huang et al., 1997).

In this study, we sought to identify and characterize the N-terminally truncated HER-2/neu protein and examine its correlation with ECD shedding and association with breast cancer pathologic factors.

MATERIALS AND METHODS

Cells and Antibodies. Cell lines were obtained from the American Type Culture Collection (Rockville, MD) except the 3T3 cells transfected with HER-2/neu cDNA, 17-3-1, were provided by Applied bioTechnology, Inc. (Cambridge, MA) and the human mammary epithelial cells (HMEC) provided by Dr. Gary Shipley of Portland, OR were cultured from tissue obtained from reduction mammoplasty. Antipeptide antibody against the C-terminus of p185HER-2/neu, anti-neu(C), has been previously described (41). Monoclonal antibody against the extracellular domain of HER-2/neu was prepared as described (42) and was provided by Applied bioTechnology Inc.

Cell Culture. 17-3-1 cells, were cultured in Dulbecco's modified Eagles medium (DMEM) supplemented with 5% fetal bovine serum containing 0.4 mg/ml geneticin (G418 GIBCO-BRL). The human breast carcinoma cell line BT474 was cultured in RPMI medium supplemented with 10% FBS and 10 μg/ml insulin. All other cell lines were grown in DMEM supplemented with 10% FBS and the antibiotic gentamicin at 0.05%.

Immunoprecipitations and Immune Complex Kinase Assays. Freshly prepared cell lysates in TEDG buffer (50 mM Tris, 1.5 mM EDTA, 0.5mM dithiothreitol , 10% glycerol pH 7.5 with 1% aprotinin, 2mM PMSF, and 2 mM vanadate) containing 1% Nonidet P-40 were immunoprecipitated by incubation with antibody for 2 hrs with continuous shaking at 4° C as described (Lin & Clinton, 1990). The immune complexes, bound to Protein G Sepharose (Pharmacia), were washed twice with TEDG buffer and incubated 10 min on ice in a kinase reaction mixture containing 20 mM HEPES pH 8.0, 2 mM dithiothreitol, 25 μM vanadate, 0.5%

Nonidet P-40, 10 mM MnCl2, 1 μM ATP, and 15 μCi (α-32P)ATP (New England Nuclear). The immune complexes were washed 3 times with buffer and the proteins were released by boiling for 2 min in SDS-PAGE sample buffer.

Western Blotting. Following SDS-PAGE, cell lysates or proteins from concentrated, conditioned medium were electroblotted onto nitrocellulose (Trans-Blot, Bio-Rad) using a semi-dry transfer unit (Bio-Rad) at 15 volts for 20 min per mini gel of 0.75 mm thickness (Mini-PROTEAN II electrophoresis cell, BioRad) equilibrated with 25 mM Tris pH 8.3, 192 mM glycine, 50 mM NaCl, 20% methanol. Binding sites were blocked by incubating the membrane with 5% nonfat dry milk. After incubation with the primary antibody, the blot was washed twice for 15 min and 4 times for 5 min with Tris-buffered saline (TBS) containing 0.05% Tween and then incubated for 40 min with goat anti-rabbit or goat anti-mouse antibody conjugated to horseradish peroxidase (HRP) (Bio-Rad) diluted in TBS-Tween. After incubation with secondary antibody, the blot was washed as described above with TBS-Tween and developed with chemiluminescent reagent (Pierce).

Cancer Tissue Extraction and Fractionation. About 0.1 gm of tumor tissue, which had been fresh-frozen and stored at -70° C, was minced on dry ice and suspended in TEDG buffer. Tissues were homogenized using a Brinkman polytron for 5-10 second bursts repeated 2-3 times with a chilled probe. Homogenates were centrifuged at 1500 x g for 10 min at 4° C. The lipid layer was removed with a wooden stick and the supernatant was centrifuged for 20 min at 40,000 x g at 4° C. The lipid layer was collected with a wooden stick, the supernatant decanted, and the pellet containing the membranes was solubilized in TEDG buffer containing 0.1%SDS for 20 min with intermittent vortexing and clarified by centrifugation at 15,000 x g for 15 min. The protein concentration in the supernatant was determined by the Bio-Rad protein assay reagent and aliquots were frozen at -80oC.

Analysis of P95 and P185 in Breast Cancer Tissues. Twenty μg of protein from the membrane fraction prepared from each tumor sample were resolved under denaturing and reducing conditions by SDS-PAGE in 10% gels. Each gel also contained 3 μg of protein from extracts of 17-3-1 cells to mark the migration of p185 and p95 and to provide a standard for the entire study. Proteins were electrotransferred onto membranes as described above, which were incubated with anti-neu(C) diluted 1:10,000 in TBS-Tween at 4° C overnight with shaking and then incubated with a 1:10,000 dilution of goat anti-rabbit HRP conjugated antibody (Bio-Rad) for 40 min at room temperature. To develop the blot, the membranes were incubated with chemilumenescent reagent (Pierce) for 5 min and then exposed to Kodak X-OMAT AR film for 1, 5, 20, and 120 min. To define the samples that overexpressed p185HER-2/neu, specimens with HER-2 immunoassay values that were considered HER-2/neu-rich (400 units or greater) compared to samples with low HER-2/neu levels (less than 400 units) were characterized for their p185 signal relative to the control 17-3-1 cells by western analysis. Those samples with a p185 signal that could be detected by 1 min exposure of the membrane to film and that was equal to or greater than the p185 level found in 3 μg of 17-3-1 cells, as revealed by laser densitometric analysis of the film, were scored as highly positive. Using this method, we identified 21.7% of the samples that overexpressed p185. This proportion is comparable to the 15-30% of breast cancers found to overexpress HER-2/neu in numerous clinical studies (5-10). In the samples that had detectable p95, its level ranged from 10% to 100% of p185. In this pilot study, specimens were scored as positive if p95 was detected at a 10% or greater proportion of p185 by 2 hrs of exposure of the membrane to film. Because of the high titer of the primary antibody, anti-neu(C), there were rarely any background bands even when the immunoblots were exposed to film for 2 hrs.

HER-2/neu Tissue Extract ELISA. Aliquots of membrane-rich fractions prepared from breast cancer tissue as described above were assayed using the Triton Diagnostics c-erbB-2 Tissue Extract EIA kit (Ciba Corning) according to manufacturer's instructions. This assay

employs two monoclonal antibodies against the HER-2/neu ECD. The HER-2/neu units/mg protein in the specimens were calculated from a calibration curve generated by plotting the HER-2/neu concentration of the calibration standards versus the absorbance obtained from the immunoassay.

Clinical Data. This investigation of human tissues was reviewed and approved by the Institutional Review Board Committee on Human Research. A computer database contains clinical information on each patient, coded to protect the individual's identity, and includes age, nodal status, size of the primary tumor, age of the patient, stage of disease at diagnosis, estrogen receptor (ER) levels and progesterone receptor (PR) receptor levels. Specimens were considered ER positive and PR positive if they contained at least 10 fmol specific binding sites per mg of cytosolic proteins.The stage of the specimens included 1 at stage 0, 32 at stage I, 56 stage II, 45 stage III and 13 stage IV. Fourteen were of unknown stage. The average age of the patients was 60. The 8 ovarian cancer tissues included 3 that were grade III and 5 that were grade IV.

RESULTS

Identification of N-terminally truncated HER-2/neu protein with kinase activity. 3T3 cells transfected with HER-2/neu cDNA (17-3-1 cells) release soluble ECD by proteolytic processing of p185HER-2/neu (Zabrecky et al., 1991). To detect truncated cytoplasmic products, 17-3-1 extracts were resolved in gels and immunoblotted with antibodies against the C-terminus of the HER-2/neu product [anti-neu(C)]. Two major protein products were detected in cell extracts; the full length p185 HER-2/neu and a truncated protein of about 95 kDa (Fig.1, lane 1). Extracts were immunoprecipitated and the 95kDa protein as well as p185HER-2/neu were phosphorylated in the immune complex with (α-32P)ATP (Fig.1, lane 2). A monoclonal antibody specific for the N-terminal region of p185HER-2/neu [anti-neu(N)] did not immunoprecipitate p95 indicating that the N-terminal region was missing (lane 3). To examine whether p95 had self-phosphorylating activity or was a substrate of the full length receptor tyrosine kinase, p185 was first removed from the cell lysate with anti-neu(N), and then p95 was

immunoprecipitated with anti-neu(C). P95 was phosphorylated when p185 levels were greatly depleted (Fig.1 lane 4) indicating that it has kinase activity.

P95 kinase activity is in human breast carcinoma cells but not in nontumorigenic breast epithelial cells. The breast carcinoma cell line, BT474, known to release soluble ECD (Lin & Clinton, 1991) also contained two autophosphorylated HER-2/neu products, p185 and p95, which were at elevated levels compared to the nontumorigenic breast epithelial cell line HBL-100 (Fig. 2). It was possible that p95 could not be detected in the small amount of HBL-100 cells, since they express low levels of HER-2 (Kraus et al., 1987). To compensate for different levels of HER-2/neu expression (Kraus et al., 1987), the amounts of extract from HBL-100, human mammary epithelial cells, (HMEC), and three breast carcinoma cell lines were adjusted and proteins were phosphorylated with (α-32P) ATP. P95 was detected in the low (MDA-MB-453) and high (BT474 and SKBR3) HER-2/neu expressing breast carcinoma cells, but not in the HBL-100 nor HMEC cells, despite a robust signal from the HER-2/neu receptor which migrated as a slightly smaller protein in the breast epithelial cells (Fig. 2).

P95 is tyrosine phosphorylated and in the membrane fraction from BT474 cells. Tyrosine phosphorylation of tyrosine kinase receptors indicates their activation in signaling (Hynes & Stern, 1994; Dougall et al., 1994). The tyrosine phosphorylation of p95, and its subcellular location were examined by fractionation of BT474 cell extracts into a soluble fraction and a particulate fraction which were immunoprecipitated with anti-neu(C) and then subjected to western blot analysis using monoclonal antibodies against phosphotyrosine. Figure 3 illustrates that tyrosine phosphorylated p95 fractionated with p185 in the particulate fraction which contains the plasma membranes. P95 was further shown to be tyrosine phosphorylated by first immunoprecipitating with anti-phosphotyrosine antibodies and then probing the western blot with anti-neu(C) (data not shown).

P95 corresponds to levels of soluble ECD released from different cells. To examine the relationship of p95 to soluble ECD, their levels were compared in different cells under varied conditions. The basal levels of ECD and cellular p95HER-2/neu were first examined in two cell lines that overexpress HER-2/neu, BT474 and the ovarian carcinoma cell line SKOV-3, which was previously reported to produce low levels of ECD (Pupa et al., 1993). The amount of p95 relative to p185 and to cell protein was greatly elevated in BT474 cells and correspondingly, the ECD in the extracellular medium from BT474 cells, detected with anti-neu(N), was enhanced by greater than 10-fold compared to the SKOV3 cells (Fig.4).

Shedding of several membrane proteins is rapidly and transiently induced by phorbol ester tumor promoters (Ehlers et al., 1991; Hooper et al., 1997). While short term treatment with tumor promoters does not induce HER-2 shedding (Vecchi et al., 1996), chronic administration of the phorbol ester TPA synergizes with chloroquine to stimulate release of soluble HER-2 (Christianson, Lin, and Clinton, unpublished observations). To determine whether p95 and ECD were coordinately regulated, TPA (500 nM) and chloroquine (50 μM) or the control vehicle were added to the culture media of BT474 and SKOV3 cells. At 24 hrs, the ECD levels in the extracellular media and p95 levels in the cell extract were analyzed. Soluble ECD was elevated several fold in the conditioned medium from stimulated BT474 cells and SKOV3 cells, while p95 was upregulated about three-fold in BT474 cells (Fig.4). Overexposure of the immunoblot revealed that p95 in SKOV3 cell extracts was also stimulated about three-fold by TPA and chloroquine (data not shown).

A metalloprotease inhibitor depresses levels of p95 and ECD from BT474 cells. The shedding of diverse transmembrane proteins has been found to be inhibited by hydroxamic acid-based compounds, which are potent metalloproteinase inhibitors (McGeehan et al., 1994; Mohler et al., 1994; Arribas et al., 1996). We therefore tested the effects of different concentrations of the hydroxamic acid, TAPI (Mohler et al., 1994) on shedding of HER-2/neu

ECD and on cell levels of p95. TAPI at 0 to 40 μM was added to cultured BT474 cells for 24 hrs, the ECD in concentrated conditioned media was analyzed by immunoblotting with anti-neu(N), and p95 and p185 were examined in cell extracts using anti-neu(C). The results in Figure 5 show that production of ECD was partially inhibited at 1 μM TAPI and maximally inhibited by 10 μM TAPI. A residual amount of about 10% of the ECD resisted inhibition by 40 μM TAPI. The level of truncated p95 in the cytoplasm was also inhibited by TAPI, with little or no effect at 1 μM and maximal inhibition at 10 μM (Fig.5). In three separate experiments, 1 μM TAPI inhibited ECD and p95 levels by 50% or less, and in all cases, maximum inhibition was achieved by 10 μM of TAPI. No change in p185HER-2/neu levels could be detected in cells treated with TAPI or when shedding was stimulated by TPA and chloroquine (Fig.4) probably because proteolytic processsing of p185 is constitutive and limited with about 20% converted into soluble HER-2/neu in 2 hrs (Pupa et al., 1993).

Detection of p185 and p95 HER-2/neu in breast cancer tissue. Tumor tissues were homogenized, fractionated, and examined for HER-2/neu proteins by western analysis. The membrane-enriched but not the soluble fraction (data not shown) from some tumor tissues contained the full length product, p185, and the truncated p95HER-2/neu protein that comigrated with HER-2/neu proteins from the control 17-3-1 cells (Fig. 6). In addition, p95, along with p185, was detected in 2 of 8 ovarian cancer tissues (data not shown). Initial analyses of several breast cancer tissues revealed distinct expression patterns of p95 and p185. One group had no detectable p185 or p95 (see # 39 and 69, Fig. 6). A second category of specimens expressed both p185 and p95 (#60,53,04,22). An additional group contained p185 with relatively little or no p95 (#40,58,38,57,17,75). As observed in previous studies, some samples were p185-rich (#04,22,57,17,75). The samples that were characterized as highly positive for p185 were initially

identified by immunoassay values of greater than 400 units (see Methods and legend to Fig. 6). The results of the Western analysis suggested that the tumors were heterogeneous with respect to HER-2/neu protein products and that they could be subdivided based on the presence or absence of p95.

Western analysis of 161 breast cancer samples revealed that 22.4% were p95 positive. The p185 positive samples were further subdivided into highly positive or HER-2-rich specimens based on comparisons with HER-2/neu overexpressing samples identified by immunoassay and comparisons with the control 17-3-1 extract as described in Methods. The highly positive p185 samples represented 21.7% of the total. All samples that expressed p95 were also positive for p185, although 65% of p185 positive samples did not contain p95. Of the p95 positive samples, 63.9% were also highly positive for p185 and 36% had low p185 levels.

Relationship between p95 positive, p185 highly positive, and other prognostic factors. Of 78 node-positive breast cancer patients, a higher proportion expressed p95, than for the node negative patients (P=.032), while p185 rich samples had no significant association with node status (Table 1).

Neither p95 positive nor p185 rich samples correlated significantly with other factors known to predict poor prognosis (McGuire et al., 1992) including estrogen receptor and progesterone receptor negativity or tumor size of 3 cm or greater (Table 1).

Influence of p95 in the p185 highly positive group. We questioned why a similar percentage of node positive and node negative patients were p185-rich (24.4% versus 22.2%, Table 1), while p95 was associated with node positive patients, since 65.7% of the p185-rich samples contained p95. We therefore examined whether the presence or absence of p95 in the specimens that overexpressed p185HER-2/neu affected the relationship with lymph node status (Table 2). The p185 highly positive samples that contained p95 (n=21) had a significantly higher association with metastasis to the lymph nodes, while the p185 highly positive samples that were negative for p95 (n=11) were associated with lymph node negative patients (P=.017).

DISCUSSION

We identified an N-terminally truncated HER-2/neu product of about 95 kDa, which was detected by western blotting and by immunoprecipitation with anti-peptide antibodies against the C-terminus, but did not react with monoclonal antibodies against the N-terminus of p185HER-2/neu. P95 has kinase activity evidenced by its self-phosphorylation when p185 was cleared from the cell extract prior to immunoprecipitation with anti-neu(C) (Fig.1). Several controls and extraction procedures were conducted to rule out that p95 was created by an *in vitro* degradation artifact. Cells extracted with protease inhibitors had only two major cytoplasmic HER-2/neu proteins, p95 and p185, with no indication of smaller degradation products. P95 levels were not affected by procedures that would eliminate the activity of proteases including direct extraction of cells in boiling 10% SDS-containing buffers (Christianson and Clinton, data not shown).

One mechanism previously described for generation of N-terminally truncated receptor tyrosine kinases is by proteolytic release of their ECD (Downing et al., 1989; Cabrera et al., 1996; O'Bryan et al., 1995; Vecchi et al., 1996). Several lines of evidence point to the production of p95HER-2 in cultured cells by endoproteolytic processing. Its presence in 17-3-1 cells transfected with HER-2/neu cDNA argues that p95 is a proteolytic product rather than the product of an alternative transcript. Furthermore, the levels of p95 and soluble HER-2 ECD released from cultured cells were correlated. First, both p95 and ECD levels were low in SKOV3 cells compared to BT474 cells (Fig. 4). Secondly, augmentation of both p95 and ECD by long term (24hr) treatment with TPA and chloroquine (Fig.4) further indicated that the truncated HER-2 products were generated through a common pathway. Although the mechanism for this stimulation was not examined, long term exposure of cells to TPA has been found to enhance internalization of RTKs (Seedorf et al., 1995), while chloroquine, an agent that alters the pH in endosomes and lysosomes, may inhibit complete proteolytic breakdown or alter RTK trafficking (Marshall, 1985). Finally, p95 and ECD were both inhibited by addition of the hydroxamate compound, TAPI, to intact cells

and both were maximally inhibited by 10 μM or less of TAPI (Fig. 5). The strong inhibition by TAPI indicates that most of the ECD and p95 in BT474 cells were generated by a metalloprotease (McGeehan et al., 1994; Mohler et al., 1994) and that this class of inhibitors may be effective in controlling shedding in breast cancer patients. Although p95 and shedding were modulated under several different conditions, changes in the p185 levels could not be detected. Unlike several transmembrane proteins that only shed when induced by TPA, proteolytic shedding of p185 occurs continually at a low basal level (Lin & Clinton, 1991; Zabrecky et al., 1991) with only about 20% converted into soluble ECD in 2 hrs (Pupa et al., 1993). The truncated cell protein of about 95 kDa described here was somewhat larger than the expected 75-80 kDa for the cytoplasmic remnant of the ~105-110 kDa ECD. P95 or the ECD might migrate anomalously in gels, since the site of cleavage for ECD shedding is not known. While our studies showed that the ECD and p95 are coordinately produced in culture by proteolytic activity that is sensitive to a metalloprotease inhibitor, it is not yet known whether p95 levels in breast tumors will be directly coupled to serum ECD. In some cases ECD may be the product of an alternative transcript (Scott et al., 1993), or the metabolism of p95 may vary in different cells. Future studies aimed at testing cancer tissue and serum from the same patients will be required to evaluate whether serum ECD correlates with tissue p95 *in vivo.*

A HER-2/neu product of the same size, 95kDa, in transfected 3T3 cells, cultured breast carcinoma cells, breast cancer tissue, and ovarian cancer tissue suggests that a similar proteolytic processing event may occur in the different cells. However p95 was not detected in all cells and tumor tissue that contain p185. Two nontumorigenic breast epithelial cell lines had no detectable p95 (Fig.2). In addition, the SKOV3 ovarian carcinoma cells, which overexpress p185, had a disproportionately low amount of p95 (Fig.4). These observations indicate that production of p95 is regulated. The cells with variable levels of truncated HER-2/neu products may differ in the

amount of the relevant protease activity or the protein substrate may have an altered conformation affecting sensitivity to proteolytic cleavage.

P95HER-2/neu has properties that suggested a rationale for examining its association with prognostic factors in breast cancers. It has kinase activity, is tyrosine phosphorylated suggesting its activity in signaling, and is truncated from its N-terminus. Oncogenic signaling by HER-2/neu is known to depend on its level of kinase activity (DiFiore et al., 1987; Hudziak et al., 1987; Segatto et al., 1988). Since p95 was at 100% of p185 in some breast cancer samples, it may impact the amplitude of the kinase signal. Moreover, an N-terminally truncated kinase domain such as p95 is expected to emit a constitutive signal by analogy to results with engineered deletions of the ECD from the HER-2/neu product (Vecchi et al., 1997; DiFiore et al., 1987; Hudziak et al., 1987; Segatto et al., 1988; Bargman & Weinberg, 1988). Taken together these results suggest that p95 will elevate the kinase signal in some patients and could thereby be associated with more aggressive tumor growth. While this was the biological rationale used in our study, p95 could also mark tumors that express a particular protease that is relevant to breast cancer pathology.

In this study, 161 breast cancer tissues were homogenized, fractionated and analyzed by western blotting, a technique required to distinguish p185 from the truncated cytoplasmic protein. A comprehensive study conducted by Tandon et al,.(1989) also used western analysis of breast tissue extracts, but they evaluated only the full length product, p185. In agreement with their results, we also found p185 expressed frequently in breast tumors with a subpopulation of 21.7% compared to their 16% that was scored as highly positive. Western blot technique has been reported to be the most prone to false-negative errors because of dilutional artifacts introduced by extracellular matrix from stroma-rich cancers (Cabrera et al., 1996). For our study, homogenized tissue was fractionated into a membrane-rich fraction from which relatively insoluble extracellular matrix proteins would tend to be eliminated.

One of the major findings of this study is that breast cancers, which express HER-2/neu, are heterogeneous with respect to protein products. Importantly, the distinct products, p95 and

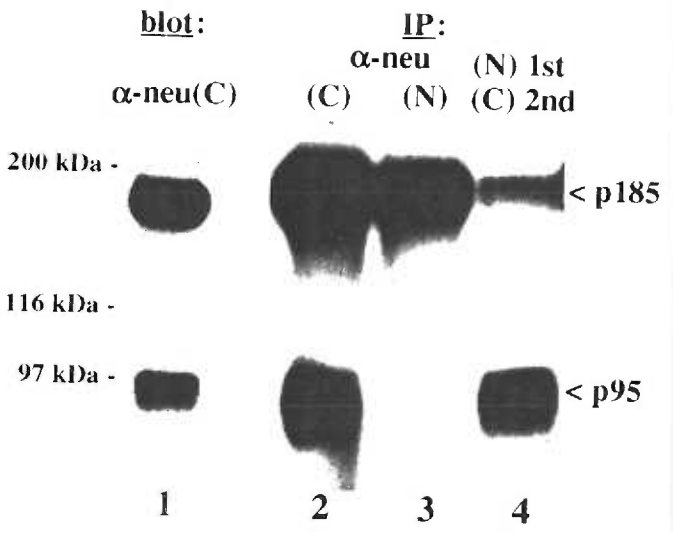
p185, were differentially associated with node status. While the group that overexpressed p185 did not associate with node status (Table 1), those that were p185-rich and contained p95 were significantly associated with lymph node metastasis (Table 2). This may help explain why several previous studies, which have attempted to show association with lymph node metastasis based on assays of p185 protein overexpression or HER-2/neu gene amplification, have yielded inconsistent results (see Singleton & Strickler, 1992). A biological explanation for our findings is that loss of the ECD regulatory region from the p95 kinase combined with amplified p185 signal in primary breast tumor cells could promote their metastasis to the lymph nodes.

P95 positive or p185 highly positive samples did not correlate with other prognostic markers in this study including tumor size or hormone receptor status. While no consistent correlation with tumor size has been detected, other studies have reported association of HER-2/neu overexpression with ER and PR negativity (Singleton & Strickjler, 1992; Tandon et al., 1989; Carlomango et al., 1996). The smaller numbers of samples examined in our studies may explain the failure to reach statistical significance. Moreover, in contrast to our study, the relationship between HER-2 overexpression and hormone receptor status was examined in a subgroup of high risk patients or in groups that were stratified by levels of hormone receptors (Tandon et al., 1989; Carlomango et al., 1996).

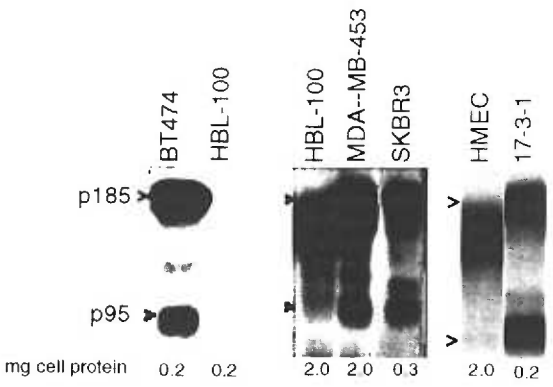
In conclusion, it has been difficult to understand why HER-2/neu overexpression occurs in tumor tissue from both node negative and positive patients, yet it is a strong prognostic marker only in node positive patients (Slamon et al., 1989, 1987; Singleton & Strickler, 1992; Press et al., 1990; Tandon et al., 1989). Our findings, which need to be validated in a different patient population, indicate that p95 is preferentially found in HER-2/neu positive patients with lymph node involvment. It is possible that higher expression of p95 is a critical factor that helps explain the increased prognostic significance of HER-2/neu in node positive patients. It will be of interest and importance to examine the prognostic significance of p95 particularly in node negative patients.

Chapter 4 Figures

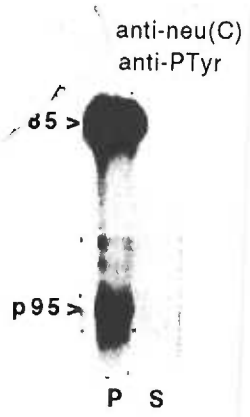
ch. 4, Fig. 1. N-terminally truncated HER-2/neu product with kinase activity. 25 μg protein from 17-3-1 cells were western blotted with anti-neu (C) diluted 1:10,000 (lane 1). In lanes 2-4, 400 μg protein were immunoprecipitated with anti-neu(C) (lanes 2,4) or with monoclonal antibody against the extracellular domain, anti-neu(N) (lane 3), or depleted of p185HER-2/neu by extracting twice with anti-neu(N) and then immunoprecipitated with anti-neu(C) (lane 4). The immune complexes were phosphorylated with (α32P) ATP and analyzed by SDS-PAGE and autoradiography.


blot:
IP:
α-neu
(N) 1st
α-neu(C)
(C)
(N)
(C) 2nd
200 kDa -
< p185
116 kDa -
97 kDa -
< p95
1
2
3
4

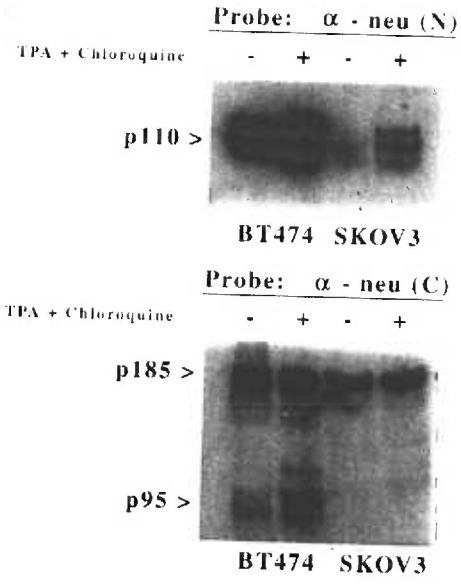
ch. 4, Fig. 2. Human breast carcinoma cell lines contain p95HER-2/neu. Indicated amounts of cell lysates from BT474, HBL-100, MDA-MB-453, SKBR3, HMEC, and 17-3-1 cells were immunoprecipitated with anti-neu (C) and phosphorylated as in Fig 1.


BT474
HBL-100
HBL-100
MDA-MB-453
SKBR3
HMEC
17-3-1
p185
p95
mg cell protein
0.2
0.2
2.0
2.0
0.3
2.0
0.2

ch. 4, Fig. 3. Tyrosine phosphorylation of p95 localized in the particulate fraction of BT474 breast carcinoma cells. Particulate (P) and soluble (S) fractions were prepared by incubation of 107 cells in ice for 10 min in 3 ml of 10 mM Tris pH7.4, 10 mM NaCl, 2 mM MgCl2 with 2 mM vanadate and protease inhibitors, Dounce homogenization, and centrifugation at 100,000 x g for 1 hr. The pellet was resuspended in 3 ml of homogenization buffer, and 200 μg of protein from the particulate fraction and an equal volume of the soluble fraction were immunoprecipitated with anti-neu(C) and analyzed as a western blot with monoclonal anti-phosphotyrosine antibody (Sigma).

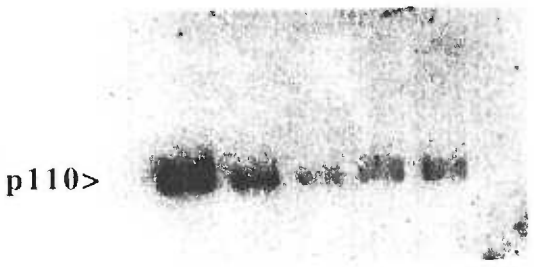

anti-neu(C)
anti-PTyr
p95
P
S

ch. 4, Fig. 4. Expression of p95 and ECD in SKOV3 and BT474 cells. Cells were treated for 24 hrs in serum-free medium with control vehicle or with 500 nM of the phorbol ester TPA and 50 μM chloroquine. In the top panel, 5 ml of conditioned media was concentrated 100 fold, denatured under nonreducing conditions, and aliquots normalized to cell extract protein were analyzed by western blotting with anti-neu(N) monoclonal antibody at 1 μg/ml. In the lower panel, 20 μg of cell proteins were analyzed by western blotting using anti-neu(C). The results are representative of three replicate experiments.


Probe: α - neu (N)
TPA + Chloroquine
- + - +
p110 >
BT474 SKOV3
Probe: α - neu (C)
TPA + Chloroquine
- + - +
p185 >
p95 >
BT474 SKOV3

ch. 4, Fig. 5. P95 and ECD are inhibited by the hydroxamic acid, TAPI. BT474 cells in serum-free medium were treated for 24 hrs with the control vehicle or with 1, 10, 20, and 40 μM TAPI (a gift from Immunex). In the top panel, the concentrated, conditioned media, normalized to the amount of cell extract, were analyzed by western blotting with anti-neu (N). Similar results were obtained when 5 μg of protein from the conditioned media from each culture were analyzed. In the lower panel, 20 μg of cell proteins were analyzed by western blotting using anti-neu(C).

Probe: α - neu (N)



0 1 10 20 40

TAP I (μM)

Probe: α-neu (C)

0 1 10 20 40

TAP I (μM)

ch. 4, Fig. 6. Western blotting analysis of 12 breast cancer tissues. Human intraductal breast cancer tissues were fractionated and 20 μg of protein from 12 patients were subjected to western blotting with anti-neu(C) as described in Methods. The control lane contained 3 μg protein from transfected 3T3 cells, 17-3-1. The position of p185, the top band, and p95 the lower band, are marked in the control 17-3-1 sample in the lower panel. The top panel is a photograph of the film that was exposed to the membrane for 20 min and the bottom panel was exposed for 5 min. HER-2/neu immunoassay values were: <100 Units for #60,39,69; 389 U for #40; 258 U for #58; 302 U for #38; 200 U for #53; 2000 U for #04; 10,000 U for #22; 1000 U for #57; 550 U for #17; 674 U for #75.

Probe: anti-neu (C)

Patient # (Cont) 60 40 58 38 53 39

(cont) 69 04 22 57 17 75

Table 1 *Relationship between p95-positive and highly p185-positive status and other prognostic factors*[a]

Factor	% p95 positive	P	% p185 highly positive	P
Nodes		.032		NS[b]
Positive (78)	29.5		24.4	
Negative (63)	14.3		22.2	
Tumor size (cm)		NS		NS
≥3 (54)	27.8		22.2	
<3 (79)	17.7		21.5	
ER		NS		NS
Negative (37)	32.0		29.7	
Positive (117)	19.7		17.9	
PR		NS		NS
Negative (59)	23.7		20.3	
Positive (95)	22.1		23.2	

[a] 161 samples were examined by Western analysis. See "Materials and Methods" for a description of patient material used and methods of analysis. Not all samples had information for the factors examined.

[b] NS, not significant.

Table 2 *Relationship between highly p185-positive samples that are p95 negative versus p95 positive and node status*

	Highly p185 positive[a]	
	p95 positive (n = 21)	p95 negative (n = 11)
Node positive	71.4%[b]	27.3%
Node negative	28.6%	72.7%

[a] The highly p185-positive group (n = 32) was divided into those that contained p95 (n = 21) and those that were p95 negative (n = 11).

[b] The samples that contained p95 had a significantly higher association with node-positive patients (15 of 21), and those that were p95 negative correlated with node negative patients (8 of 11; P = 0.017).

Chapter 5

Thesis Discussion:

Summary and Conclusions

This work describes the characterization of three alternative HER-2/neu gene products: (1) the ECDIIIa intron-containing HER-2 transcript that encodes Herstatin, (2) an alternative 8 kb HER-2 mRNA, and (3) a proteolytically-activated HER-2 tyrosine-kinase, p95. This discussion will describe my hypotheses for the role and possible clinical utility of these unique HER-2/neu oncogene products in human cancer.

Identification of p68ECDIIIa and protein product, "Herstatin"

The first section will address the novelty of the HER-2 ECDIIIa gene product, Herstatin, regarding its origin, structure, and function, and will include speculations about significance and implications for its function in normal development and future clinical utility.

Origin of the ECDIIIa-containing HER-2 mRNA

The ECDIIIa mRNA, which encodes Herstatin, is an alternative transcript of novel structure. It is transcribed by a unique mechanism of single intron retention from the unaltered HER-2/neu gene. That the retention of an apparent intronic sequence occurs in a functional mRNA without evidence of any point mutations in the HER-2 gene sequence is, in itself, unusual. A large body of literature exists citing incidents of intron retention resulting in human disease. In the vast majority of cases, the splicing aberrance's have resulted from single or multiple base-pair mutations at the genomic level (reviewed in Krawczak et al., 1992). Additionally, the protein products resulting from such splicing aberrance's are largely nonfunctional. The insertion of intronic sequences may result in loss of function by introducing shifts in reading frame or premature translation termination, either of which can result in deletion of functional domains, destabilization of the mRNA, or

destabilization of the protein product. Thus, the finding described here underscores the potential functional significance of specific intron sequences within genomic DNA.

Structure of ECDIIIa HER-2 mRNA

The 274 nucleotide retained intron, determined to be intron 8 by homology with EGF-R and HER-3 gene structures, is internal. Therefore, its retention results in an extraordinarily large, functional exon of greater than 500 nucleotides, comprising exon 8, intron 8, and exon 9. The average exon length has been estimated to be 154 bp in a large survey of vertebrate gene sequences (Berget, 1995). Variation in exon length exhibits a bell-shaped distribution with less than 1% of all exons being greater than 500 bp. Because intron 8 does not contain a consensus polyadenylation signal, the alternative transcript must utilize a downstream polyadenylation signal, that may be either from a retained terminal intron, as in the 2.3 kb transcript reported (Benz, 1993), or the terminal HER-2 exon of the 4.5 kb HER-2 transcript that encodes p185. The last exon, which contains 3'UTR sequence, exhibits no size constraints and can be up to several kb in length (Berget, 1995). Thus, the resulting known alternative transcripts are either 2.6 kb or 4.8 kb in size. The unique protein product of either of these transcripts is predicted to be identical, due to a termination codon within the retained intron 8 sequence.

ECDIIIa mRNA Generation

The mechanism for intron retention is unclear, as little is known about regulation of RNA splicing mechanisms. In fact, considerable debate still exists over whether splicing occurs by recognition of exon sequences or of intron sequences (Berget, 1995; Neel et al., 1993). However, evidence exists for the presence of silencing factors that may act to protect introns from splicing during production of alternative splice forms (Phelan et al., 1996; Philips et al., 1998). Possibly, a

nuclear protein exists to regulate splicing by binding nascent RNA molecules at or near splice recognition sequences and, thereby, blocks splicing machinery.

Regulation of ECDIIIa mRNA

In comparison with the 4.5 kb mRNA, the ECDIIIa-containing transcript may be expected to have decreased stability, due to the presence of a premature translation termination codon (Beelman & Parker, 1995; Jacobsen & Peltz, 1996; Standart & Jackson, 1994). Therefore, the expression of this transcript may be tightly controlled by multiple mechanisms, including regulation of its stability.

The expression pattern of this alternative mRNA shows tissue selectivity in the human fetus (ch.2, Fig. 2) and suggests the possibility of developmental regulation. Northern analysis of human fetal tissues revealed detectable expression levels only in kidney and liver of the 4.8 kb and 2.6 kb alternative HER-2 transcripts described above, respectively.

In contrast, expression of this alternative transcript is repressed in human carcinoma cell lines with increased HER-2 gene copies (ch. 2, Figs. 4 & 5). The more sensitive ribonuclease protection assay (RPA) was neccessary to detect its expression in three carcinoma cells lines with HER-2 gene amplification and p185HER-2 overexpression, in comparison to the nontumorigenic human embryonic kidney cell line, HEK-293. The RPA shows significantly lower expression levels, at less than 5% of the 4.5 kb transcript in carcinoma cell lines, compared to about 25% in HEK-293 cells. This further suggests that tumor cells with gene amplification may have a mechanism for downregulating expression of the alternative transcript. This could occur by squelching factors required for retaining intron 8 (i.e., a nuclear factor that inhibits intron 8 splicing).

Characterization of p68ECDIIIa, Herstatin

Importantly, the intron-containing transcript is expressed as a stable protein (ch. 2, Fig. 3). The protein product, p68ECDIIIa, which we have named Herstatin, is novel in that it contains a unique C-terminal sequence attached to an amino-terminal portion of the p185HER-2 receptor itself. The intron-encoded sequence specifies the unique carboxyl-terminus of the protein identified as the product of the alternative ECDIIIa HER-2 mRNA, p68ECDIIIa. This C-terminal ECDIIIa sequence of 79 amino acids is unlike any protein sequence entered in the protein databases of Genbank and Entrez (Blast). It is unusually rich in proline residues, comprising 19% of amino acids in this C-terminal region. The ECDIIIa amino acid sequence also contains a stretch of hydophobic residues about 22 amino acids in length, though the extent of hydrophobicity does not predict a transmembrane domain. Together, these attributes give clues towards its possible structure, predicted to contain a looped helix.

Interestingly, p68ECDIIIa contains the amino-terminal regions encoded by the 4.5 kb transcript, including the signal sequence for entry into the secretory pathway. With identical sequence to p185 extracellular subdomains I and II as its amino-terminus, the p68ECDIIIa contains the five N-linked glcosylation sites encoded in these sequences, as well as 27 cysteine residues. In addition, there is a consensus N-linked glycosylation site within the intron-encoded sequence at the carboxy-terminus. Glycosylation events may be important in the final structure and, possibly, function of the protein. Indeed, the bacterially-produced protein, which is not glycosylated, appeared to have an unstable structure compared to the naturally-occurring p68ECDIIIa from conditioned media.

p68ECDIIIa Function

The structure of p68ECDIIIa provides suggestions of function, which were investigated in my studies.

p68ECDIIIa Directly Binds p185HER-2 as a Unique Ligand

As a ligand, p68ECDIIIa is unique in several respects. First, it is the first reported RTK ligand that is encoded by the receptor gene itself by alternative splicing. Second, it contains amino-terminal subdomains I and II of identical sequence to p185HER-2. It is not unprecedented for RTK genes to encode truncated protein forms that have identical amino-terminal sequence and result from alternative RNA processing. The gene for the fibroblast growth factor receptor, FGF-R, produces several alternatively spliced transcripts that encode secreted forms of the receptor which bind FGFs *in vitro* with different affinities (Vainikka et al., 1992; Werner et al., 1992; Yayon et al., 1992). Similarly, the EGF-R gene produces a secreted form (Petch et al., 1990; Ullrich et al., 1984;), which inhibits receptor activation (Basu et al., 1989). In addition, several other RTK genes encode secreted forms of the receptors, including the genes for HER-3 (Lee et al., 1998; Katoh et al., 1993), platelet-derived growth factor receptor (PDGF-R)(Vu et al., 1989), insulin-like growth factor receptor II/Mannose 6-Phosphate Receptor (MacDonald et al., 1989), and the interleukin-4 (IL-4) receptor (Mosley et al., 1989). However, the protein products of these alternative splice forms do not contain unique intron-encoded C-terminal sequences, do not form stable complexes with the receptor, nor bind with nanomolar affinity.

Alternative transcripts encoding RTK ectodomains, particularly the ECD of EGF-R and HER-2, have been proposed to function in an antagonistic manner, blocking receptor dimerization (Petch et al., 1990; Basu et al., 1989; Scott et al., 1993). However, ECDs without a transmembrane anchor or other stabilizing structure (e.g., IgG-Fc domains), do not form stable interactions *in vitro* (Fitzpatrick et al., 1998; Horan et al., 1995). In contrast, alternative FGF-R and EGF-R ectodomains bind FGFs and EGFs, respectively, and have thus been proposed to exert a negative regulatory effect by inhibiting ligand-mediated receptor activation (Vainikka et al., 1992; Werner et al., 1992; Petch et al., 1990; Basu et al., 1989).

Another distinguishing feature of p68 as a ligand is that it does not contain an EGF domain. However, other unique RTK ligands have recently been identified that also lack an EGF domain. For example, collagen is a extracellular matrix protein that is also rich in proline residues. Collagen has recently been identified as a mammalian Discoidin Domain Receptor (DDR) family RTK ligand (Vogel et al., 1997). Collagen was shown to directly bind with high affinity and activate the orphan receptors DDR1 and DDR2.

Model for the Function of p68ECDIIIa

Herstatin contains sequences of p185HER-2 extracellular subdomains I and II, but has an alternative domain at the location of the cysteine-poor subdomain III, which is the precise region of putative high-affinity ligand binding in p185HER-2 by homology with the EGF-R. Because p185HER-2 is proposed to homodimerize in a ligand-independent manner, it is plausible that the subdomains I and II of the full-length receptor interact within the dimer. Interestingly, the ECDs of EGF-R and HER-2 have been shown to form heteromeric interactions with the full-length receptors when co-expressed (O'Rourke et al., 1998). Alternative transcripts encoding the ECD of EGF-R and HER-2 have been proposed to function in an antagonistic manner, blocking receptor dimerization (Petch et al., 1990; Basu et al., 1989; Scott et al., 1993). However, ECDs without a transmembrane anchor or other stabilizing structure (e.g., IgG-Fc domains), do not form stable interactions *in vitro* (Fitzpatrick et al., 1998; Horan et al., 1995). In accordance with these previous observations, our binding studies indicate that the ECDIIIa domain confers binding to p185HER-2 but because the Kd is smaller for p50ECDIIIa than p17 (data not shown), these results further suggest that the interaction may be facilitated or stabilized by the HER-2 extracellular subdomains I and II within p68ECDIIIa.

Effects of p68ECDIIIa on Anchorage-independent Growth

Since p68ECDIIIa displays HER-2-selective binding and inhibition of growth, we have named it Herstatin. The Herstatin effects we observed in anchorage-independent growth assays best implicate its role in carcinogenesis. Both Herstatin from HEK- 293 cells (i.e., in HEK-293 CM) and bacterially-expressed p50ECDIIIa showed inhibition of the growth of HER-2-transformed cells in soft agar. In fact, the protein appeared to induce apoptosis of HER-2 overexpressing cells, without an observable effect on *src*527-transformed cells.

Both Recombinant and Native Herstatin Rapidly disrupt HER-2 dimers

When we examined effects of both Herstatin in HEK-293 CM (i.e., native) and bacterially-expressed p50ECDIIIa (i.e., recombinant) on HER-2 homodimers in 17-3-1 cells, a treatment time course revealed a rapid inhibition of HER-2 dimers at the cell surface by ten minutes (ch. 2, Fig. 8). Decreased tyrosine phosphorylation in dimers was concordantly observed (data not shown).

Considering the reported turnover time for cell-surface HER-2 receptors, about 4-12 hours, this rapid effect on HER-2 dimers cannot be due to inhibition of new dimer formation. Rather, it indicates a disruption of existing dimers. Since dimers are proposed to be in a state of dynamic equilibrium, it is possible that Herstatin might bind monomers and disrupt the equilibrium. Alternatively, disruption of dimers could result from internalization and degradation of ligand-bound receptors.

Furthermore, the Herstatin response in 17-3-1 cells displayed an apparent desensitization effect, wherein dimers gradually reappeared to baseline levels by 24 hours of treatment. A likely reason for this observation is that the bacterially-expressed p50ECDIIIa protein may have been

destabilized or oxidized in serum-free media, since we observed intermolecular dissulfide cross-linking of this protein *in vitro* in the absence of dithiotreitol (DTT).

Implications of Herstatin Expression in Development

Although a function has not been ascribed for HER-2 in normal development, evidence implicates HER-2 as a mediator of growth and of metastatic behavior in cancer (Tan et al., 1997; Xu et al., 1997; Yu et al., 1994, 1993), suggesting a possible role for HER-2 in mediating similar functions in normal tissues during development. We detected abundant levels of Herstatin protein in the human embryonic kidney cell line, HEK-293, and expression of Herstatin mRNA in human fetal kidney and liver, where its role in down-regulating p185HER-2 may be essential. From our findings, we speculate that the Herstatin expression pattern suggests tissue-specific developmental downregulation of p185HER-2 activity. It is also possible that Herstatin could downregulate HER-2-mediated progenitor cell growth and proliferation. Herstatin may play a temporal role in "turning-off" p185HER-2 activity during development. There is evidence that downregulation of p185HER-2 is required during development, since transgenic mice engineered to express the activated form of p185HER-2 died early, from one day to within 16 weeks of birth. Early death was apparently due to hyperplasia of several organs, resulting in organ dysfunction and was especially evident in kidney and lung tissues (Stocklin et al., 1993; Suda et al., 1990).

To explore the role of Herstatin in development, it would be of interest to study Herstatin knockout animals, generated by deleting the intron 8 sequence from both alleles of the HER-2/neu gene. However, first the homologous 8th intron of the *neu* gene must be cloned and sequenced from mice genomic DNA to ensure the presence of a mouse homologue of Herstatin. The rat *neu* intron 8 was cloned in our laboratory (Joni Doherty, unpublished results), and the sequence did not indicate a Herstatin homologue. Mice likely do not express Herstatin either, so a mouse knockout would not be useful.

Implications of Herstatin for Human Cancer

As the first described natural antagonist of p185HER-2, Herstatin holds potential as an anti-cancer therapeutic. The fact that carcinoma cells exhibiting HER-2 gene amplification express relatively low levels of Herstatin compared to p185HER-2 suggests a mechanism, which may be mediated by gene amplification, to override the negative regulatory effects of Herstatin on p185HER-2 activity. Thus, by adding back Herstatin to the extracellular milieu in cancer patients whose tumors exhibit p185HER-2 overexpression, inhibition of tumor progression may be achieved. This could be attained by intravenous infusion of purified Herstatin from mammalian cells. Furthermore, this protein would be less likely to induce an immune response in the host because it is native. Moreover, if the Herstatin effects we have seen in anchorage-independent growth assays are applicable to whole tumors, Herstatin might induce apoptosis and, thus, lead to tumor regression.

Proposed Future Studies of Herstatin

(1)*Studies of Herstatin purified from a eukaryotic expression system*

Ultimately, to more thoroughly investigate and verify our results, it will be neccessary to produce Herstatin in eukaryotic cells for purification of the glycosylated, properly-folded form. This can be achieved by employing transfection methods in heterologous systems which impart N-linked glycosylation. We are addressing this issue currently using HeLa cells.

First, it will be imperative to repeat anchorage-independent growth studies using either purified recombinant Herstatin or transfect cells with a Herstatin expression vector. We can then compare their behavior in soft agar with that of mock-treated or mock-transfected cells (or cells transfected with a C-terminally truncated form of the Herstatin that lacks the ECDIIIa domain).

Since Herstatin disrupted p185HER-2 dimers in 17-3-1 cells, which may be homodimers, it may have a similar effect on heterodimers. It will be an important next step to examine effects on heterodimer formation in SKBR-3 and MCF-7 cells. Because HER-3 is kinase inactive (Pinkas-Kramarski et al., 1996; Riese et al., 1995), yet undergoes heregulin-induced homo- and heterodimerization (Graus-Porta et al., 1997; Karunagaran et al., 1996; Sliwkowski et al., 1994), its dimerization with HER-2 can be measured by examining HER-3 tyrosine phosphorylation in response to heregulin (Schaefer et al., 1999; Graus-Porta et al., 1997; Karunagaran et al., 1996). MCF-7 cells provide an ideal model for such experiments, as they express abundant amounts of the kinase-inactive HER-3 and relatively low levels of HER-2, HER-4, and HER-1. SKBR-3 cells are another appurtenant model since they express abundant amounts of HER-2 and HER-3, but have undetectable levels of the other EGF-R family members.

(2)*Determine if Herstatin binds other class I RTKs*

One unanswered question of importance is whether Herstatin binds other EGF-R family members. To examine this, a pull-down assay with histidine-tagged p50ECDIII could be conducted. This assay was successfully used to show specific binding of the novel intron–encoded peptide, ECDIIIa, to p185HER-2. Another method of assessing binding would be to determine whether ^{125}Ip50ECDIII directly binds to the EGF-R or other family members by conducting cross-linking reactions as described in chapter 2.

If other EGF-R family member(s) bind Herstatin, the next step would be to determine whether binding elicits similar effects on dimers or whether its effects are limited to HER-2. This analysis could be conducted on cells ectopically expressing selected EGF-R family members and analyzing effects of Herstatin on dimers, as described for 17-3-1 (ch.3). If Herstatin binds to other EGF-R family members, then it would expected to have a similar effect on their dimers. However,

because of the instrically high kinase activity of HER-2, one might suspect that Herstatin acts as a specific inhibitor of only HER-2 homodimers.

(3) *Determine whether the intron-encoded sequence, ECDIIIa, elicits a biological response*

The ECDIIIa novel peptide also displays specific binding to p185HER-2 (data not shown). It would be of interest to determine whether this binding is sufficient to achieve a biological response similar to that of p50ECDIII. The effects of p17ECDIIIa on HER-2 dimers in 17-3-1 and anchorage-independent growth should be compared with p50ECDIIa in these assays. Since the ECDIIIa domain may confer p185HER-2 binding, and binds p185HER-2 itself, it would be expected to have some independent effect, which may actually be to compete with Herstatin for binding of p185HER-2. Thus, the ECDIIIa domain alone may block the response to Herstatin. It is difficult to speculate, though, on whether HER-2 monomers with bound ECDIIIa domain proteins would dimerize, since the mechanism for HER-2 homodimerization is poorly understood.

(4) *Investigate the mechanism by which Herstatin affects HER-2 dimers*

Our initial results demonstrate that Herstatin blocks HER-2 dimers. If Herstatin is an antagonist, it should block downstream signaling by p185HER-2. This could be initially examined by determining the profile of tyrosine phosphorylated cellular proteins before and after Herstatin treatment. Some signal transducing factors which have been shown to link HER-2 RTK activation to MAP kinase activation, such as Shc or Grb2, would be expected to exhibit decreased tyrosine phosphorylation in response to Herstatin treatment. Investigation of MAP kinase activation or inhibition by measuring substrate level phosphorylation, of myelin basic protein, for example, in a kinase assay would also be important.

(5) *Assess whether Herstatin blocks tumorigenesis*

To determine whether Herstatin suppresses tumorigenesis, nude mice could be injected subcutaneously or intraperitoneally with HER-2 transformed cells, such as 17-3-1 or SKOV-3. The nude mice harboring these tumors could then be treated intravenously with purified recombinant Herstatin. Herstatin levels in serum could be monitored to estimate clearance and tumor formation would be assessed and/or measured over time to score Herstatin effectiveness.

(6) *Explore the regulatory mechanism for splicing of intron 8 from the HER-2 gene*

To decipher the proposed splicing regulatory mechanism, it may be necessary to investigate splicing regulatory factors and study their activity in different cells. One possibility is to conduct a toeprint analysis on mRNA containing the intron. This method could identify the presence and location of RNA binding proteins. It can be done with *in vitro* synthesized ECDIIIa transcript, which might first be incubated with nuclear extracts from HEK-293, for example, since these cells would expected to contain such a factor, and from a cell line that does not express Herstain, for comparison.

An alternative method for identifying RNA binding proteins is the electromobility shift assay (EMSA, band shift or gel shift assay), which can be done in the conventional manner using radiolabelled *in vitro* synthesized ECDIIIa RNA, rather than a radiolabelled DNA probe.

- **An Alternative 8 kb HER-2/neu mRNA**

Like the ECDIIIa alternative transcript, the 8 kb alternative mRNA is transcribed from an unaltered HER-2/neu gene, but by a different mechanism. This transcript is produced by DNA polymerase II read- through of a consensus polyadenylation signal in the terminal HER-2 exon (ch. 3, Figs. 3 & 6) Further, a second consensus polyadenylation site was found in the sequence of the extended 3'UTR that was also not utilizedfor production of this transcript. The characterization of the 8 kb

transcript resulted in determination of additional sequence that extends the known HER-2 gene sequence boundaries, defines the location of additional polyadenylation signals, and suggests an alternative mechanism for enhanced levels of HER-2 expression in human cancer.

8 kb HER-2 mRNA Expression

The aggressive ovarian cancer cell line, SKOV-3 expresses the 8 kb HER-2 transcript even more abundantly than the conventional 4.5 kb. Few other ovarian cell lines overexpress HER-2. Although the 8 kb HER-2 mRNA has not been found in any of these, it could be below the levels of detection. It is also possible that the 8 kb mRNA is expressed in ovarian cancers, since few primary human ovarian tumors have been examined at the mRNA level. While we have been unable to detect the 8 kb transcript in Northern analysis of fetal tissues, it could again be expressed below the levels of detection. Therefore, although no additional tissues or cell lines have been identified that express an 8 kb alternative transcript, we cannot yet rule out its presence in other cell lines and tissues.

Is the 8 kb mRNA translated?

Investigations of HER-2 protein products in SKOV-3, which express abundant amounts of the 8 kb transcript, have revealed no alternative protein product resulting from this unusually large transcript. Rather, it appears that selection for expression of the 8 kb mRNA is based on its enhanced intrinsic stability. That the 8 kb HER-2 mRNA is both polyadenylated and exhibits increased stability suggest that it could be translated into p185HER-2. Untranslated mRNAs, or mRNAs with premature translation termination, have been shown to be unstable (reviewed in Surdej et al., 1994).

Future studies should be directed toward determining whether the 8 kb transcript is translated. A polysome profile could be conducted as an assessment of translation initiation rate. Furthermore, a polysome profile comparing the 8 kb and 4.5 kb transcripts would indicate whether the extended 3'UTR of the 8 kb might facilitate translation. Evidence suggests that sequences or secondary structure of the 3'UTR in mRNA, by providing sites for RNA-binding protein association, might regulate rate of translation initiation by facilitating interactions between poly(A)-binding protein (PABP) and the 5'methyl-7-guanine cap structure (Hake & Richter, 1997; Jackson, 1993). Another method would be *in vitro* translation of the transcript, from an expression vector construct containing the cDNA sequence of the entire 8 kb transcript.

Additionally, to test whether the 8 kb transcript is translated, the cDNA could be transfected as a mammalian expression vector into a cell line that lacks HER-2 expression, such as Ba/F3. Further studies of the expression of the 8 kb with that of the 4.5 kb expression could be conducted to compare the intrinsic stability and translation of these two transcripts.

Possible Functions of the 3'UTR Extension in the 8kb mRNA

The 3'UTR, like the coding region of mRNA, contains encrypted information. In contrast to coding sequence, the complex codes within 3'UTR determine precise regulation of gene expression and have yet to be completely deciphered. Some of the information may be structural, involving RNA secondary structure determined by primary sequence of the 3'UTR, which creates specific protein binding sites. The hexanucleotide AAUAAA has long been recognized as a nuclear polyadenylation signal (Hake & Richter, 1997), but we are just beginning to recognize common sequence elements that confer mRNA stability, cytoplasmic poly(A) extension, subcellular localization, translation initiation, and rapid degradation.

Perhaps the most thoroughly studied of these functions is 3'UTR-mediated mRNA stability. Sequence elements have been identified, acting in cis within the 3'UTR, that confer rapid degradation of some mRNAs (Asson-Baters et al., 1994; Shaw & Kamen, 1986). Other elements may confer stability, but these appear to be more complex and specific to RNA-protein interactions (Zhou et al., 1998; Jackson, 1993). The overall emerging theme is that mRNA stability can be conferred by properties that are determined by the secondary structure within its 3'UTR. This may involve stabilizing stem-loop structural formations or binding of an mRNA stabilizing protein.

The expression of the 8 kb HER-2 is reminiscent of developmental or maternal mRNAs which sometimes differ in 3'UTR length and/or sequence from that of their adult counterparts (Hake & Richter, 1997). The difference in 3'UTRs between the 4.5 and 8 kb HER-2 transcripts might confer an altered rate of nuclear export, subcellular localization and/or temporal expression (Jackson, 1993). Although neither of the known elements that confer such regulation, a 60 nt sequence (Braun, 1991) and the translation inhibition element (TIE)(Robbie, 1995), were found in the 8 kb 3'UTR sequence obtained in a fractionated cDNA library clone, the clone is incomplete. Therefore, these possibilities have not yet been completely investigated.

Implications and Future Studies of 8 kb HER-2 mRNA

The expression of the 8 kb alternative HER-2 mRNA demonstrates regulation of polyadenylation site choice. Because this transcript contains two skipped consensus hexanucleotide sequences within its 3'UTR, it could provide a means for studying the mechanism regulating nuclear polyadenylation site choice and mapping the sequences that might confer this process. Such regulatory mechanisms may be important in developmental gene expression.

It would be of interest to investigate whether selective deletion of the 8 kb HER-2 transcript alters the potent oncogenicity of SKOV-3. Possible methods include the use of antisense oligonucleotides or ribozymes designed to specifically target the extended 3'UTR. For oligos, depending on the region of 3'UTR to which they are complimentary, the resulting effect could be further stabilization of the 8 kb. Therefore, 8 kb mRNA stability would need to be assessed in response to an array of antisense oligos. Use of ribozymes is an alternative method of selectively targeting the 8 kb transcript for degradation. The growth of SKOV-3 in soft agar or invasive behavior could be assayed to score the effects of altering 8 kb HER-2 expression. An alternative method to assess the function of the 8 kb HER-2 transcript would be to transfect its cDNA into exogenous cells. Thus, an elaborate investigation of the behavior of cells, in which expression of the 8 kb HER-2 transcript is controlled, might elucidate its function.

- **p95: Function and Significance**

The presence of the proteolytically-cleaved HER-2 ECD in the serum of cancer patients suggested a constitutively active kinase existing as the cytoplasmic remnant of p185HER-2. We have investigated this possibility in breast carcinoma cell lines and primary tumors. We detected an N-terminally truncated HER-2 protein of 95 kDa in cell lines that shed the soluble ECD, and have determined it to have kinase activity. Importantly, we detected p95HER-2 in primary breast tumor tissues and found it to be significantly correlated with metastasis to lymph nodes (chapter 4).

Because conventional clinical assays used to detect HER-2 overexpression have employed antibodies directed against the N-terminal or extracellular portion of p185HER-2, either by enzyme-linked immunosorbent assays (ELISA) or by immunohistochemistry, p95HER-2 expressing tumors were not previously detected. Such oversights could contribute to the lack of

clinical utility of p185HER-2 overexpression to predict outcome in node-negative patients (Singleton & Strickler, 1992). In this group, the need for a prognostic marker is most pressing, since 30% will relapse (Mansour et al., 1997).

The HER-2 ECD levels in serum of breast cancer patients has been shown to correlate directly with tumor load (Molina et al., 1996; Brandt-Rauf, 1995). Therefore, its usefulness as a prognostic indicator is limited because its release from a small tumor may go undetected, due to serum dilutional factors. Our results indicate that the event of HER-2 ECD shedding, resulting in active p95 expression, may define a subpopulation of highly aggressive tumors. Thus, the same tumor that sheds ECD may express p95 at levels that are easily detectable by Western analysis of a lumpectomy specimen. The level of p95 expression correlates directly with ECD levels in tissue culture and might also in breast cancer patients. Because it is not subject to the variable dilutional effects of circulation volume, it could be a better prognostic indicator. Also, p95 expression may be useful for staging tumors since it shows statistically significant association with lymph node metastasis.

Further, shedding provides a mechanism for evading immunosurveillance, which is based on aberrant overexpression of p185HER-2 as a marker at the tumor cell surface. In fact, in the Herceptin clinical trials, breast cancer patients whose tumors exhibited ECD shedding showed either no response to Herceptin or experienced tumor progression which correlated with increased ECD in serum (). This suggests that ECD shedding confers resistance to Herceptin and raises the possibility that shedding tumors are more aggressive. It was also noted in the phase II and III trials that serum antibody titers quickly declined below therapeutic levels in patients expressing the soluble ECD, indicating that the HER-2 ECD neutralizes Herceptin antibodies in serum. This is not surprising since the recognition sequence of the monoclonal Herceptin antibody (rhuMAb4D5) has been mapped to residues 529-625 of p185HER-2, sequence which is within the shed HER-2

ECD found in the serum of breast cancer patients. Hence, the shedding event and p95 remnant would likely confer resistance to any cancer treatments that target the HER-2 ECD.

p95: Regulation of Expression

Shedding is an apparent metalloprotease-mediated cleavage event, that is inhibited by TAPI, an hydroxamate metalloprotease inhibitor, in tissue culture models. The specificity of this inhibitor raises the potential for an anti-HER-2 therapeutic in the form of a metalloprotease inhibitor, like TAPI, that could work in combination with Herceptin (and/or Herstatin).

The HER-2 ECD has also been reported as a product of an alternatively spliced HER-2 RNA transcript. It is still unclear, however, whether the ECD product of the alternative 2.3 kb transcript is secreted, as predicted, since it has not been detected in conditioned media from cells that express the truncated mRNA (Scott et al., 1993). In a recent study, the level of this transcript detected by RT-PCR was increased relative to the full-length 4.5 kb HER-2 transcript in lymph node and bone metastases from breast cancers (Gebhardt et al., 1998). However, the design of the RT-PCR analysis and interpretation was such that detection of the ECD-encoding transcript expression would include transcripts of 2.6 kb with a retained intron 8. Because this was not considered and Western blot analysis was not used to examine HER-2 protein products from the tissues analyzed, these tissues could be expressing Herstatin rather than HER-2 ECD.

p95: Future Directions

The significance of p95 warrants further investigation as a marker of metastasis and to determine whether its expression directly correlates with serum ECD. It will be important to determine if p95 has predictive value in node negative breast cancer. This could be investigated using early staged

(I & II) breast cancer specimens, assessing p95 expression by Western analysis, and obtaining sufficient clinical follow-up data for 5-10 years.

In summary, the current model of HER-2 oncogenic potency needs revision to incorporate important alternative forms of the HER-2 gene products that we have characterized in the Clinton laboratory.

REFERENCES

Adams, C.L., Nelson, W.J., and Smith, S.J. Quantitative analysis of cadherin-catenin-actin reorganization during development of cell-cell adhesion. *J. Cell Biol.* 135 (6), 1996.

Alimandi M., Romano A., Curia M.C., Muraro R., Fedi P., Aaronson S.A., Di Fiore P.P., Kraus M.H. Cooperative signaling of ErbB3 and ErbB2 in neoplastic transformation of human mammary carcinoma cells Oncogene 15:1813-1821 (1995).

Arai, Y., Yoshiki, T., and Yoshida, O. C-erbB-2 oncoprotein: a potential biomarker of advanced prostate cancer. *Prostate, 30*: 195-201, 1997.

Arribas, J., Coodly, L., Vollmer, P., Kishimoto, T.K., Rose-John, S., and Massague, J. Diverse cell surface protein ectodomains are shed by a system sensitive to metalloprotease inhibitors. *J. Biol. Chem. 271*: 11376-11382, 1996.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K. (editors). *Current Protocols in Molecular Biology*. Vol.1, Supplement 9 (1990), 2.1.1-2.1.3. Massachusetts General Hospital, Harvard Medical School: Green Publishing Associates and Wiley-Interscience.

Baasner S., Melchner H.V., Kleener T., Hilgard P., and Beckers T. Reversible tumorigenesis in mice by conditional expression of the HER-2/cerbB-2 receptor tyrosine kinase. Oncogene 3:901-911 (1996).

Bargmann, C.I., and Weinberg, R.A. Oncogenic activation of the neu-encoded receptor protein by point mutation and deletion. *EMBO J., 7:* 2043-2052, 1988.

Baselga, J., Tripathy, D., Mendelsohn, J., Baughman, S., Benz, C.C., Dantis, L., Sklarin, N.T., Seidman, A.D.D., Hudis, C.A., Moore, J., Rosen, P.P., Twaddell, T.T., Henderson, I.C., and Norton, L. Phase II study of weekly intravenous recombinant humanized anti-p185HER2/neu-overexpressing metastatic breast cancer. *J. Clin. Oncol., 14*: 737-744, 1996.

Basu, A., Raghunath, M., Bishayee, S., and Das, J. (1989) Inhibition of tyrosine kinase activity of the epidermal growth factor (EGF) receptor by a truncated receptor form that binds to EGF: role for interrectpro interaction in kinase regulation. *Mol. Cell. Biol.* **9,** 671-677.

Baulida J. Kraus M.H., Alimandi M.,Di Fiore P.P. and Carpenter G. All ErbB receptors other than the epidermal growth factor receptor are endocytosis impaired. J. Biol. Chem. 271:5251-5257 (1996).

Beelman, C.A. & Parker, R. Degradation of mRNA in eukaryotes. *Cell* **81**, 179-183 (1995).

Ben-Levy, R., Paterson, H.F., Marshall, C.J., and Yarden, Y. A single autophosphorylation site confers oncogenicity to the neu/erbB-2 receptor and enables coupling to the MAP kinase pathway. *EMBO J.* **13**(14), 3302-3311 (1994).

Beerli R.R. and Hynes N.E. Epidermal growth factor-related peptides activate distinct subsets of erbB receptors and differ in their biologial activities. J. Biol. Chem. 271:6071-6076 (1996).

Benz, C.C., Scott, G.K.Sarup, J.C. Estrogen-dependent, tamoxifen-resistant tumorigenic growth of MCF-7 cells transfected with HER-2/neu. Breast Cancer Res. Treat. 24:85-95, 1993.

Brandt-Rauf, P.W. The c-erb transmembrane growth factor receptors as serum biomarkers in human cancer studies. *Mutation Res., 333*: 203-208, 1995.

Braun, R.E. Temporal regulation of the protamine 1 gene during mouse spermatogenesis. *Enzyme* **44**, 120-128 (1991).

Brodowicz, T., Wiltschke, C., Budinsky, A.C., Krainer, M., Steger, G.G., and Zielinski, C.C. Soluble HER-2/neu neutralizes biologic effects of anti-HER-2/neu antibody on breast cancer cells in vitro. *Int. J. of Cancer., 73*: 875-879, 1997.

Brodowicz, K.M., Zeillinger, R., Wiltschke, C., Scholten, C., Seifert, M., Kubista, E., and Zielinski, C.C. Tissue expression and serum levels of HER-2/neu in patients with breast cancer. *Oncology, 54*: 475-481, 1997.

Cabrera, N., Diaz-Rodriguez, E., Martin-Zanca, D., and Pandiella, A. TrkA receptor ectodomain cleavage generates a tyrosine-phosphorylated cell-associated fragment. *J. Cell. Biol., 132:* 427-436, 1996.

Cao, J. & Geballe, A. P. Coding sequence-dependent ribosomal arrest at termination of translation. *Molec. Cell. Biol.* 16(2), 603-608 (1996).

Carlomagno, C., Perrone, F., Gallo, C., DeLaurentiis, M., Lauria, R., Morabito, A., Pettinato, G., Panico, L., D'Antonio, A., Bianco, A.R., and De Placido, S. c-erbB2 overexpression decreases the benefit of adjuvant tamoxifen in early-stage breast cancer without axillary lymph node metastases. *J. Clin. Oncol., 14*: 2702-2708, 1996.

Carraway, K. L. and Cantley L. A neu acquaintance for ErbB3 and ErbB4: a role for receptor heterodimerization in growth signaling. *Cell* 78:5-8 (1994).

Cohen, B.D., Kiener, P.A., Green, J.M., Foy, L., Fell, P., and Zhang, K. The relationship between human epidermal growth-like factor receptor expression and cellular transformation in NIH3T3 cells. *J. Biol. Chem.* 271(48), 30897-30903 (1996).

Cohen, G.B., Ren R., Baltimore D. Modular binding domains in signal transduction proteins. *Cell* 80:237-248 (1995).

Cohen, J.A., Weiner, D.B., More, K.F. Kodai, Y., Williams, W.V., Maguire, H.C.J., LiVolsi, V.A., and Greene, M.I. Expression pattern of the neu (NGL) gene-encoded growth factor receptor protein (p185neu) in normal and transformed epithelial tissues of the digestive tract. *Oncogene*, *4*: 81-88, 1989.

Coussens, L., Yang-Feng, T.L., Liao, Y.C., Chen, E., Gray, A., McGrath, J., Seeburg, P.H., Libermann, T.A., Schlessinger, J., Francke, U., Levinson, A., and Ullrich, A. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. *Science*, *230:* 1132-1139, 1985.

Culp, P.A. and Musci, T.J. Translational activation and cytoplasmic polyadenylation of FGF receptor-1 are independently regulated during Xenopus oocyte maturation. *Dev. Biol.* **193**, 63-76 1998).

Deshane, J., Cabrera, G., Grim, J.E., Siegal, G.P., Pike, J., Alvarez, R.D., and Curiel, D.T. Targeted eradication of ovarian cancer mediated by intracellular expression of anti-erbB-2 single-chain antibody. *Gynecologic Oncology* **59**, 8-14 (1995).

DiFiore, P.P., Pierce, J.H., Kraus, M.H., Segatto, O., King, C.R. and Aaronson, S.A. ErbB-2 is a potent oncogene when overexpressed in NIH3T3 cells. *Science* **237**, 178-182 (1987).

Dougall, W.C., Qian, X., Peterson, N.C., Miller, M.J., Samanta, A., and Greene, M.I. The neu-oncogene: Signal transduction pathways, transformation mechanisms and evolving therapies. *Oncogene* **9**, 2109-2123 (1994).

Downing, J.R., Roussel, M.F., and Sherr, C.J. Ligand and protein kinase C downmodulate the colony-stimulating factor 1 receptor by independent mechanisms. *Mol. Cell .Biol.* , *9*: 2890-2896, 1989.

Earp, H.S., Dawson T.L., Li X., and Yu H. Heterodimerization and functional interaction between EGF receptor family members: A new signaling paradigm with implications for breast cancer research. *Breast Cancer Res. Treat.* 35:115-132 (1995).

Ehlers, M.R., and Riordan, J.F. Membrane proteins with soluble counterparts: role of proteolysis in the release of transmembrane proteins. *Biochemistry, 30:* 10065-10074, 1991.

Fehm, T., Maimonis, P., Katalinic, A., and Jager, W.H. The prognostic significance of c-erbB-2 serum protein in metastatic breast cancer. *Oncology, 55*: 33-38, 1998.

Felip, E., Del Campo, J.M., Rubio, D. Overexpression of c-erbB-2 in epithelial ovarian cancer: Prognostic value and relationship with response to chemotherapy. *Cancer* 75: 2147-2152 (1995).

Fitzpatrick, V.D., Pisacane P.I, Vandlen R.L., and Sliwkowski M.X. Formation of a high affinity heregulin binding site using the soluble extracellular domains of ErbB2 with ErbB3 or ErbB4. *FEBS Letters* 431:102-106 (1998).

Fruendlieb, S., Baron U., Bonin A.L., Gosen M., Bujard H. Use of tetracycline-controlled gene expression systems to study mammalian cell cycle. Meth. in Enzym. 283:157-173 (1997).

Fujimoto, J., Hori, M., Ichigo, S., Misao, R., and Tamaya, T. Preliminary study of oncogene expressions in endometrial cancers: Aberrant estrogen receptor gene and erbB-2 expressions. *Europ. J. Gyn. Onc.* **16**(1), 40-47 (1995).

Gebhardt, F., Zanker, K.S. and Brandt, B. Differential expression of alternatively spliced c-erbB-2 mRNA in primary tumors, lymph node metastases, and bone marrow micrometastases from breast cancer patients. *Biochem Biophys Res. Comm.* 247, 319-323 (1998).

Givol D. and Yayon A. Complexity of FGF receptors: genetic basis for structural diversity and functional specificity. *FASEB J.* 6:3362-3369 (1992).

Graus-Porta D., Beerli R.R, Daly J.M., and Hynes N.E. ErbB-2, the preferred heterodimerization partner of all Erb receptor is a mediator of lateral signaling. *EMBO J.,* 16:1647-1655 (1997).

Groenen L.C., Nice E.C., and Burgess A.W. Structure-function relationships for the EGF/TGF-" family of mitogens. *Growth Factors* 11:235-257 (1994).

Guy, C.T., Webster, M.A., and Schaller, M. Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. *Proc. Natl. Acad. Sci. USA* **89**, 10578-10582 (1992).

Guy P.M. Platko J.V., Cantley L.C., Cerione R.A. and Carraway K.L. Insect cell-expressed p180erbB-3 possesses an impaired tyrosine kinase activity. *Proc. Natl. Acad. Sci. USA* 91:8132-8136 (1994).

Hake L.E. & Richter, J.D. Translational regulation of maternal mRNA. . *Biochem. et Biophys. Acta,* 1332 (1997) M31-M38.

Harty, R.N. & O'Callaghan, D.J. Isolation and purification of specific, overlapping mRNA species by electroelution from agarose gels. *Biotechniques* **10**(6), 728-729 (1991).

Heldin C.-H. and Ostman A. Ligand-induced dimerization of growth factor receptors: A variation on the theme. *Cytokine Growth Factor Rev.* 7:33-40 (1996).

Higashiyama S., Abraham J.A., Miller J.C., Fiddes J.C. and Klagsbrun M. A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. *Science* 251:936-939 (1991).

Hollywood, D.P. and Hurst, H.C. A novel transcription factor, OB2-1, is required for overexpression of the proto-oncogene c-*erb*B-2 in mammary tumour lines. *EMBO J.* **12**(6), 2369-2375 (1993).

Hooper, N.M., Karran, E.H., and Turner, A.J. Membrane protein secretases. *Biochem. J., 321*: 265-279 (1997).

Howes R., Wasserman J.D., and Freeman M. In vivo analysis of Argos structure-function. *J. Biol. CHem.* 273:5275-4281 (1998).

Hua, W., Christianson, T., Rougeot, C., Rochefort, H., and Clinton, G.M. SKOV3 ovarian carcinoma cells have functional estrogen receptor but are growth resistant to estrogen and antiestrogens. *J. Steroid Biochem.* **55**(3/4), 279-289 (1995).

Huang, H.-J., Nagane, M., Klingbie,l C.K., Lin, H., Nishikawa, R., JiX.-D., Huang, C.-M., Gill, G.G., Wiley, H.S. and, Cavenee, W.K. The enhanced tumorigeneic activity of a mutant epidermal growth factor receptor common in human cancers is mediated by

threshold levels of constitutive tyrosine phosphorylation and unattenuated signaling. *J. Biol. Chem. 272*: 2927-2935 (1997).

Huang, S.S., Koh, H.A., Konish, Y. Bullock, L.D., & Huang, J.S. Differential processing and turnover of the oncogenically-activated neu/erbB2 gene product and its normal cellular counterpart. *J. Biol. Chem.* 265(6), 3340-6 (1990).

Hudziak R.M., Lewis G.D., Winget M., Fendly B.M., Shepard M., and Ullrich A. p185HER-2 monoclonal antibody has antiproliferative effects in vitro and sensitizes human breast tumor cells to tumor necrosis factor. *Mol. and Cell. Biol.* 9:1165-1172 (1989).

Hudziak, R.M., Schlessinger, J., Ullrich, A . Increased expression of the putative growth factor receptor p185HER-2 causes transformation and tumorigenesis of NIH3T3 cells. *Proc. Natl. Acad. Sci., 84*: 7159-7163 (1987).

Hung, M.-C., Zhang, X., Yan, D.H., Zhang, H.-Z., He, G.-p., Zhang, T.-q., and Shi, D.-r. Aberrant expression of the c-erbB-2/neu protooncogene in ovarian cancer. *Cancer Letters* **61**, 95-103 (1992).

Hynes, N.E., Sterne, D.F. The biology of erbB-2/neu/HER-2 and its role in cancer. *Biochem. Biophys. Acta,1198*: 165-184 (1994).

Jackson, R.J. Cytoplasmic regulation of mRNA function: the importance of the 3' untranslated region. *Cell* **74**, 9-14 (1993).

Jackson, R.L. & Standart, N. Do the poly(A) tail and 3'untranslated region control mRNA translation? *Cell* **62**, 15-24 (1990).

Jones, J., Lagasse, L.D., and Karlan, B.Y. Steroid hormone independence of HER-2/neu mRNA expression in four human ovarian cancer cell lines. *Gynecol. Oncol.* **55**, 421-426 (1994).

Juhl, H., Downing, S.G., Wellstein, A., and Czubayko, F. HER-2/neu is rate-limiting for ovarian cancer growth. *J. Biol. Chem.* **272**(47), 29482-29486 (1997).

Kandl, H., Seymour, L., and Bezwoda, W.R. Soluble c-erbB-2 fragment in serum correlates with disease state and predicts for shortened survival in patients with early-stage and advanced breast cancer. *Br. J. Cancer, 70*: 739-742, 1994.

Karlan, B.Y., Jones, J., Slamon, D.J., and Lagasse, L.D. Glucocorticoids stabilize HER-2/neu messenger RNA in human epithelial ovarian carcinoma cells. *Gynecol. Oncol.* **53**, 70-77 (1994).

Karunagaran D., Tzhar E., Beerli R.R., Chen X., Graus-Porta D., Ratzkin B.J., Seger R., Hynes N.E., and Yarden Y. ErbB-2 is a common auxiliary subunit of NDF and EGF receptors: implications for breast cancer. *EMBO J.* 15:254-264 (1996).

Katoh, m., Yazaki, Y., Sugimura, T., and Terada, M. c-erbB3 gene encodes secreted as well as transmembrane receptor tyrosine kinase. *Biochem. Biophys. Res. Commun.* 192(3), 1189-1197 (1993).

Kavanaugh W.M. and Williams L.T. An alternative to SH2-domains for binding tyrosine-phosphorylated proteins *Science* 266:1862-1865 (1994).

Kern, J.A., Schwardz, D.A., Nordberg, J.E., Weiner, D.B., Greene, M.I., Torney, L., and Robinson, R.A. P185HER-2/neu expression in human lung adenocarcinoma predicts shortened survival. *Cancer Res., 50*: 5184-5191, 1990.

King, C.R., Kraus, M.T., DiFiore, P.P., Paik, S., Kasprzyk, P.G. Implications of erbB-2 overexpression for basic science and clinical medicine. *seminars in Cancer Biol.* 1, 329- 337 (1990).

Kistner A., Gossen M., Zimmerman F., Jerecic J., Ullmer C., Lubbert H., Bujard H. Deoxycycline-mediated quantitative and tissue-specific control of gene expression in transgenic mice. *Proc. Natl. Acad. Sci.* 93: 10933-10938 (1996).

Klapper L.N., Vaisman N., Hurwitz E., Pinkas-Kramarski R., Yarden Y., and Sela M. A subclass of tumor-inhibitory monoclonal antibodies to erbB-2/HER2 blocks crosstalk with growth factor receptors. *Oncogene* 14:2099-2109 (1997).

Kokai Y., Myers J.N., Wada T., Brown V.I., LeVea C.M., Davis J.G., Dobashi K., and Greene M.I. Synergistic interaction of p185c-neu and the EGF-receptor leads to transformation of rodent fibroblasts. Cell 58:287-292 (1989).

Kornfeld K. Vulvar development in *Caenorhabditis elegans*.Trends Genet. 13:55-61 (1997).

Kornilova, E.S., Taverna, D., Hoeck, W., and Hynes, N.E. Surface expression of erbB-2 protein is post-translationally regulated in mammary epithelial cells by epidermal growth factor and by culture density. *Oncogene* 7, 511-519 (1992).

Kozak, M. An analysis of vertebrate mRNA sequences: Intimations of translational control. *J. Cell Biol.* 115(4), 887-903 (1991).

Kraus, M.H., Popescu, N.C., Amsbaugh, S.C., and King, C.R. Overexpression of the EGF receptor-related proto-oncogene erbB-2 in human mammary tumor cell lines by different molecular mechanisms. *EMBO J., 6*: 605-610, 1987.

Krawczak, M., Reiss, J., and Copper, D.N. *Human Genetics* 90, 41-54 (1992).

Lax I., Fischer R., Ng C., Segre J., Ullrich A., Givol K., and Schlessinger J. Noncontiguous regions in the extracellular domainof EGF receptor define ligand-binding specificity. *Cell Regul.* 2:337-345 (1991).

Lax I., Bellot F., Howk R., Ullrich A., Givol D., and Schlessinger J. Functional analysis of the ligand binding site of EGF-receptor utilizing chimeric chicken/human receptor molecules. *EMBO J.* 8:421-427 (1989).

Lax I., Burgess W., Bellot F., Ullrich A., Schlessinger J. Localization of a major receptor-binding domain for epidermal growth factor by affinity labeling. *Mol. Cell. Biol.* 8:1831-1834 (1988).

Lee, H. & Maihle, N. J. Isolation and characterization of four altrnative c-erbB3 transcripts expressed in ovarian carcinoma-derived cell lines and normal human tissues. *Oncogene* **16,** 3243-3252 (1998).

Lee K.F., Simon H., Chen H., Bates B., Hung M.-C., and Hauser C. Requirement for neuregulin receptor erbB2 in neural and cardiac development. *Nature* 378:394-398 (1995).

Leitzel, K., Teramoto, Y., Konrad, K., Chinchilli, V.M., Volas, G., Grossberg, H., Harvey, H., Demers, L., and Lipton, A. Elevated serum c-erbB-2 antigen levels and decreased response to hormone therapy of breast cancer. *J. Clin. Oncol. ,13*: 1129-1135, 1995.

Leitzel, K., Teramoto, Y., Sampson, E., Maurceri, J., Langton, B.C., Demers, L., Podczaski, E., Harver, H., Shambaugh, S., Volas, G., Weaver, S., and Lipton, A. Elevated soluble c-erbB-2 antigen levels in the serum and effusions of a proportion of breast cancer patients. *J. Clin. Oncol., 10:* 1436-1443, 1992.

Lenferink, A.E., Pinkas-Kramarski, R., van de Poll, M.L.M., van Vugt, M.J.H., Klapper, L.N., Tzahar, E., Waterman, H., Sela, M., van Zoelen, J.J., and Yarden, Y. Differential endocytic routing of homo- and hetero-dimeric erbB tyrosine kinases confers signaling superiority to receptor heterodimers. *EMBO J.* 17(12): 3385-3397 (1998).

Lichtenstein, A., Berenson, J., Gera, J.F., Waldburger, K., Martinez-Maza, O., and Berek, J.S. Resistance of human ovarian cancer cells to tumor necrosis factor and lymphokine-

activated killer cells: Correlation with expression of HER-2/neu oncogenes. *Cancer Res.* **50**, 7364-7370 (1990).

Lin, C.R., Chen, W.S., Kruiger, W., Stolarsky, L.S., Weber, W., Evans, R.M., Verma, I.M., Gill, G., and Rosenfeld, M.G. Expression cloning of human EGF receptor complimentary DNA: gene amplification and three related messenger RNA products in A431 cells. *Science* **224**, 843-848 (1984).

Lin Y.J., Christianson T.A., Clinton G.M. Disulfide-linked and noncovalent dimers of p185HER-2/neu in human breast carcinoma cells. *J. Cell. Biochem. 49:* 290-295, (1992).

Lin, Y.J., and Clinton, G.M. A soluble protein related to the HER-2/neu proto-oncogene product is released from human breast carcinoma cells. *Oncogene, 6:* 639-643, 1991.

Lin, Y.J., Li, S., and Clinton, G.M. Insulin and epidermal growth factor stimulate phosphorylation of p185HER-2/neu in the breast carcinoma cell line, BT474. *Mol. Cell. Endocrin., 69*: 111-119, 1990.

Lonardo F., Di Marco E., King C.R., Pierce J.H., Segatto O., Aaronson S.A., and Di Fiore P.P. The normal erbB-2 product is an atypical receptor-like tyrosine kinase with constitutive activity in the absence of ligand. *New Biol* 2: 992-1003 (1990).

Lupu R., Colomer R., Zugmaier G., Sarup J., Shepard M., Slamon D., and Lippman M. Direct interaction of a ligand for the erbB2 oncogene product with the EGF receptor and p185erbB2.*Science* 249:1552-1555 (1990).

MacDonald, R.G., Tepper, M.A., Clairmont, K.B., Perregaux, S.B., & Czech, M.P. Serum form of the rat Insulin-like growth factor II/Mannose 6-phosphate receptor is truncated in the carboxyl-terminal domain. *J. Biol. Chem.* 264(6), 3256-3261 (1989).

Maden H., Marx D., Schauer A., Kuhn W. Prognostic significance of p105 (c-erbB-2 HER-2/neu) serum levels in patients with ovarian cancer. *Anticancer Res.*, *17*: 757-760, 1997.

Maisonpierre P.C., Suri C., Jones P.F., Bartunkova S., Wiegand S.J., Radziejewski C., Compton D., McClain J., Aldrich T.H., Papadopoulos N., Daly T.J., Davis S., Sato T.N., and Yancopoulos G.D. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science 277: 55-60 (1997).

Mansour, O.A., Zekri, A.R., Harvery, J., Teramoto, Y., and Elahmady, O. Tissue and serum c-erbB-2 and tissue EGFR in breast carcinoma - three years follow-up. *Anticancer Research*, *17*: 3101-3105, 1997.

Marshall, S. Kinetics of insulin receptor internalization and recycling in adipocytes. *J. Biol. Chem*, *260*: 4136-4144, 1985.

McGeehan, G.M., Becherer, J.D., Bast, R.C., Boyer, C.M., Champion, B., Connolly, K.M., Conway, J.G., Furdon, P., Karp, S., Kidao, S., and McElroy, A.B. Regulation of tumour necrosis factor-" by a metalloproteinase inhibitor. *Nature 370*:561 (1994).

McGuire, W.L. and Clark, G.M. Prognostic factors and treatment decisions in axillary-node-negative breast cancer. *N. Engl. J. Med.*, *326*: 1756-1761, 1992.

McKenzie, S.J., Marks, P.J., Lam, T., Morgan, J., Panicali, D.L., Trimpe, K.L., and Carney, W.P. Generation and characterization of monoclonal antibodies specific for the human neu oncogene product, p185. *Oncogene , 4:* 543-548, 1989.

Ming X. Bergering B.M.t. Wennstrom S., Claesson-Welsh L., Heldin . Bos J.L., Kozma S.C. Thomas G. Activation of map-kinase by a pathway independent of p21ras. *Nature* 371:426-429 (1994).

Mitra, A.B., Murty, V.V.V.S., Pratap, M., Sodhani, P., and Chaganti, R.S.K. ErbB2 (HER-2/neu) oncogene is frequently amplified in squamous cell carcinoma of the uterine cervix. *Cancer Res.* 54, 637-639 (1994).

Mohler, K.M., Sleath, P.R., Fitzner, J.N., Cerretti, D.P., Alderson, M., Kerwar, S.S., Torrance, D.S., Otten-Evans, C., Greenstreet, T., Weerawarna, K., Kronheim, S.R., Petersen, M., Gerhart, M., Kozlosky, C.J., March, C.J., and Black, R.A. Protection against a lethal dose of endotoxin by an inhibitor of tumour necrosis factor processing. *Nature*, *370*: 218-220, 1994.

Molina, R., Zanon, J.J., Filella, X., Farrus, B., Munoz, M., Latre, M.L., Pahisa, J., Velasco, M., Fernandez, P., Estape, J., and Bassesta, A.M. Utility of C-erB-2 in tissue and in serum in the early diagnosis of recurrence in breast cancer patients: comparison with carcinoembryonic antigen and CA 15.3. *Br. J. of Cancer*, *4*: 1126-1131, 1996.

Moscatello, D.K., Holgado-Madruga, M., Godwin, A.K., Ramirez, G., Gunn, G., Zoltick, P.W., Biegel, J.A., Hayes, R.L., and Wong, A.J. Frequent expression of a mutant epidermal growth factor receptor in multiple human tumors. *Cancer Res., 55*: 5536-5539, 1995.

Moscatello, D.K., Montgomery, R.B., Sundareshan, P., McDanel, H., Wong, M.Y., and Wong, A.J. Transformation and altered signal transduction by a naturally occurring mutant EGF receptor. *Oncogene, 13*: 85-96, 1996.

Mosley, B.M., Beckman, P., March, C.J., Idzerda, R.L., Gimpel, S.D., VandenBos, T., Friend, D., Alpert, A., Anderson, D., Jackson, J., Wignall, J.M., Smith, C., Gallis, B., Sims, J.E., Urdal, D., Widmer, M.B., Cosman, D., and Park, L.S. The murine interleukin-4 receptor: molecular cloning and characterization of secreted and membrane-bound forms. *Cell* 59, 335-348 (1989).

Munson P.J. and Rodbard D. Ligand: a versatile computerized approach for characterization of ligand-binding systems. Anal. Biochem. 107:220-239 (1980).

Myers R.B., Brown D., Oelschlager D.K., Waterbor J.W., Marshall M.E., Srivastava S., Stockard, C.R., Urban, D.A., and Grizzle, W.E. Elevated serum levels of p105 (ErbB-2) in patients with advanced-stage prostatic adenocarcinoma. *Int. J. of Cancer., 69:* 398-402, 1996.

O'Bryan, J.P., Fridell, Y.W., Koski, R., Varnum, B., and Liu, E.T. The transforming receptor tyrosine kinase, Axl, is post-translationally regulated by proteolytic cleavage. *J. Biol. Chem., 270:* 551-557, 1995.

Ochiai, A., Akimoto, S., Kanai, Y., Shibata, T., Oyama, T., and Hirohashi, S. c-erbB-2 gene product associates with catenins in human cancer cells. *Biochem. Biophys. Res. Comm.* 205(1), 73-78 (1994).

O'Rourke D.M., Qian X., Zhang H.-T., Davis J.G., Nute E., Meinkoth J., and Greene M.I. Trans receptor inhibition of human glioblastoma cells by erbB family ectodomains. *Proc. Natl. Acad. Sci.* 94:3250-3255.

Pegues, J.C. & Stromberg, K. Inducible antisense inhibition of erbB-2 expression reduces anchorage independent growth of ovarian carcinoma cells. *Cancer Letters* 117, 73-79 (1997).

Peles E, Yarden Y. Neu and its ligands: from an oncogene to neural factors. *Bioessays* 15:815-824 (1993).

Peles E., Ben Levy R., Tzahar E., Liu N., Wen D., and Yarden Y. Cell-tyep specific interaction of Neu differentiation factor (NDF/heregulin) with Neu/HER-2 suggests complex ligand-receptor relationships. *EMBO J.* 12:961-971 (1993).

Peles E., Bacus S.S., Koski R.A., Lu H.S., Wen D., Ogen S.G., Ben Levy R., and Yarden Y. Isolation of the neu/HER-2 stimulatory ligand: a 44 kd glycoprotein that induces differentiation of mammary tumor cells. *Cell* 69:205-216 (1992).

Peles E., Ben-Levy R., Or E., Ullrich A., Yarden Y. Oncogenic forms of the neu/HER-2 tyrosine kinase are permanently coupled to phospholipase C . *EMBO J., 10:* 2077-2086, (1991).

Pegram, M.D., Lipton, A., Hayes, D.F., Weber, B.L., Baselga, J.M., Tripathy, D., Baly, D., Baughman, S.A., Twaddell, T., Glaspy, J.A., & Slamon, D.J. Phase II study of receptor-enhanced chemosensitivity using recombinant humanized anti-p185HER-2/neu monoclonal antibody plus cisplatin in patients with HER-2/neu-overexpressing metastatic breast cancer refractory to chemotherapy treatment. *J. Clin. Oncol.* 16(8). 2659-2671 (1998).

Petch, L.A., J. Harris, V.W. Raymond, A. Blasband, D.C. Lee, and H.S. Earp. A truncated, secreted form of the epidermal growth factor receptor is encoded by an alternatively spliced transcript in normal rat tissue. *Mol. Cell. Biol.* 10(6):2973-2982 (1990).

Phelan, A., Dunlop, J., and Clements, J.B. Herpes simplex virus type 1 protein IE63 affects the nuclear export of virus intron-containing transcripts. *J. Virol.* 70(8), 5255-5265 (1996).

Pinkas-Kramarski R., Lenferink A.E.G., Bacus S.S., Lyass L., van de Poll M.L.M, K., Klapper L.Nl, Tzahar E., Sela M., van Zoelen E.J.J., and Yarden Y. The oncogenic EbrB-2/ErbB-3 heterodimer is a surrogate receptor fothe epidermal growth factor and betacellin. *Oncogene* 16:1249-1258 (1998).

Pinkas-Kramarski R., Shelly M., Glathe S., Ratzkin B.J., and Yarden Y. Neu differentiation factor/neuregulin isoforms activate distinct receptor combinations. *J. Biol. Chem.* 271:19029-19032 (1996).

Pinkas-Kramarski R., Soussan L., Waterman H., Levkowitz G., Alroy Ir. Klapper L., Lavi S.,Seger R., Ratzkin B.J., Sela M. and Yarden Y. Diversification of neu differentiation factor and epidermal growh factor signaling by combinatorial receptor interactions. *EMBO J.* 15:2552-2467 (1996).

Plowman G.D., Green J.M., Culouscou J.M., Carlton G.W., Rothwell V.M., And Buckley S. Heregulin induces tyrosine phosphorylation of HER4/p180erbB4. *Nature* 366:473-475 (1993).

Press, M.F., Jones, L.A., Godolphin, W., Edwards, C.L., Slamon, D.J. HER-2/neu oncogene amplification and expression in breast and ovarian cancers. [Review]. *Progress in Clinical & Biological Research, 354*: 209-221, 1990.

Pupa, S.M., Crepaldi, L., Gandino, S., Longati, P., and Comoglio, P. The extracellular domain of the c-erbB-2 oncoprotein is released from tumor cells by proteolytic cleavage. *Oncogene, 8:* 2917-2923, 1993.

Qian X., Dougall W.C., Fei Z., Greene M.I. Intermolecular association and trans-phosphorylation of different neu-kinase forms permit SH2-dependent signaling and oncogenic transformation. Oncogene. 10: 211-219 (1995).

Qian, X., LeVea, C.M., Freeman, J.K., Dougall, W.C., and Greene, M.I. Heterodimerization of epidermal growth factor receptor and wild-type or kinase-deficient Neu: A mechanism of

interreceptor kinase activation and transphosphorylation. *Proc. Natl. Acad. Sci.* 91, 1500-1504 (1994).

Riese D.J., Bermingham Y., van Raaj T.M., Buckley S., Plowman G.K., and Stern D.F. Betacellulin activates the epidermal growth factor receptor and erbB-4 and induces cellular response patterns distinct from those stimulated by EGF or neuregulin. Oncogene 12:345-353 (1996).

Riese D.J., van Raaju T.M., Plowman G.D., Andrews G.C., and Stern D.F. The cellular response to neuregulins is governed by complex interactions of the ebrB receptor family. Mol. Cell. BIol. 15:57704776 (1995).

Robbie, E.P., Peterson, M., Amaya, E., and Musci, T.J. Temporal regulation of the Xenopus FGF receptor in development: a translation inhibitory element in the 3'untranslated region. *Development* **121**, 1775-1785 (1995).

Rodriguez, G.A., and Park, M. Oncogenic activation of tyrosine kinases. *Curr. Opin. Genet. Dev., 4:* 15-24, 1994.

Rodriguez, G.C., Boente, M.P., Berchuck, A., Whitaker, R.S., O'Briant, K.C., Xu, F.< and Bast, R.C. The effect of antibodies and immunotoxins reactive with HER-2/neu on growth of ovarian and breast cancer cell lines. *Am J Obstet Gynecol.* 168(1), 228-232 (1993).

Ross, J.S.,& Fletcher, J.A. The HER-2/neu oncogene in breast cancer: Prognostic factor, predictive factor, and target for therapy. *Stem Cells* 16, 413-428 (1998).

Sachs, A.B. Messenger RNA degradation in eukaryotes. *Cell* **74**, 413-421 (1993).

Samanta A., LeVea C.M., Dougall W.C., Qian X., Greene M.I. Ligand and p185c-neu density govern receptor interactions and tyrosine kinase activation. Proc. Natl. Acad. Sci , 91: 1711-1715 (1994).

Savage C. Inagami T., and Cohen S. The primary structure of epidermal growth factor. J. Biol. Chem. 241:7612-7621 (1972).

Schlessinger, J. Direct binding and activation of receptor tyrosine kinases by collagen. *Cell* 91, 869-872 (1997).

Scott, G.K, Robles, R., Park, J.W., Montgomery, P.A., Daniel, J., Holmes, W.E., Lee, J., Keller, G.A., Li, W.-L., Fendly, B.M., Wood, W.I., Shepard, M., and Benz, C.C. A truncated intracellular HER2/neu receptor produced by alternative RNA processing affects growth of human carcinoma cells. *Mol. Cell. Biol.* **13**(4), 2247-2257 (1993).

Schnepp B., Donaldson T., Grumbling G., Ostrowshi S., Schweitzer R., SHilo B.-Z. and Simcox A. EGF domain swap converts a Drosophila EGF receptor activatorinto an inhibitor. Genes & Dev. 12:908-913 (1998).

Schaefer, G., Akita, R.W. & Sliwkowski, M.X. A discreet three-amino acid segment (LVI) at the C-terminal end of kinase-impaired erbB-3 is required for transactivation of erbB2. *J. Biol. Chem* 274(2), 859-866 (1999).

Schweitzer, R., Howes, R., Smith, R., Shilo, B-Z. & Freeman, M. Inhibition of Drosophila EGF receptor activation by the secreted protein Argos. *Nature* **376,** 699-702 (1995).

Seedorf, K., Shearman, M., and Ullrich, A. Rapid and long term effects of protein kinase C on receptor tyrosine kinase phosphorylation and degradation. *J. Biol. Chem., 270:* 18953-18960, 1995.

Segatto, O., King, R., Pierce, J.H., DiFiore, P.P., and Aaronson, S.A. Different structural alterations upregulate in vitro tyrosine kinase activity and transforming potency of the erbB-2 gene. *Mol. Cell. Biol., 8:* 5570-5574, 1988.

Seger R, and Krebs E.G. The MAPK signaling cascade. FASEB J. 9:726-735 (1995)

Sharp, P. A. & Burge, C. B. (1997) *Cell* **91,** 875-879.

Shibata, T., Ochiai, A., Kanai, Y., Akimoto, S., Gotoh, M., Yasui, N. Machinami, R., and Hirohashi, S. Dominant negative inhibition of the association between beta-catenin and c-erbB-2 by N-terminally deleted beta-catenin suppresses the invasion and metastasis of cancer cells. *Oncogene* 13, 883-889 (1996).

Singleton, T.P., and Strickler, J.G. Clinical and pathologic significance of the c-erbB-2 (HER-/neu) oncogene. *Pathol.Annual, 27 Pt 1*: 165-198, 1992.

Slamon, D.J., Godolphin, W., Jones, L.A., Holt, J.A., Wong, S.G., Keith, D.E., Levin, W.J., Stuart, S.G., Udove, J., Ullrich, A., and Press, M.F. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* **24**: 707-712 (1989).

Slamon D.J., Clark G.M. Amplification of c-erbB-2 and aggressive human breast tumors? Science 240: 1795-1798 (1988).

Slamon, D.J., Clark, G.M., Wong, S.G., Levin, W.J., Ullrich, A., and McGuire, W.L. Human breast cancer: Correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* **235**, 177-182 (1987).

Sliwkowski M.X. et al Coexpression of erbB2 and erbB3 proteins reconstitutes a high affinity receptor for heregulin. J. Biol. Chem. 269:14661-14665 (1994).

Sonnenburg, N. mRNA translation: Influence of the 5' and 3' untranslated regions. *Curr. Opin. Gen. Develop.* **4**, 310-315 (1994).

Stern, D. F., Hefferman, P. A. & Weinberg, R. A. (1986) *Mol. Cell. Biol.* **6,** 1729-1740.

Stocklin, E., Botteri, F., and Groner, B. An activated allele of the c-erbB-2 oncogene impairs kidney and lung function and causes early death of transgenic mice. *J. Cell Biol.* 122(1), 199-208 (1993).

Suda, Y., Aizawa, S., Furuta, Y., Yagi, T., Ikawa, Y., Saitoh, K., Yamada, Y., Toyoshima, K., and Yamamoto, T. Induction of a variety of tumors by c-erbB2 and clonal nature of lymphomas even with the mutated gene (V659E). *EMBO J.* 9(1), 181-190 (1990).

Sugawa, N., Ekstrand, A.J., James, C.D., and Collins, V.P. Identical splicing of aberrant epidermal growth factor receptor transcripts from amplified rearranged genes in human glioblastomas. *Proc. Natl. Acad. Sci. USA* **87**, 8602-8606 (1990).

Summerfield A.E, Hudnall A.K., Lukas T.J., Guyer C.A., and Staros J.V. Identification of residues of the epidermal growth factor receptor proximal to residue 45 of bound epidermal growth factor. *J. Biol. Chem.* 271:19656-19659 (1996).

Surdej. P., Reidl, A., and Jacobs-Lorena, M. Regulation of mRNA stability in development. *Annu. Rev. Genet.* 28, 263-282 (1994).

Tal, M., King, R., Kraus, M. H., Ullrich, A., Schlessinger, J. & Givol, D. (1987) *Mol. Cell. Biol.* **7**, 2597-2601.

Tan M. Yao Y. and Yu D. Overexpressionof the c-erbB-2 gene enhanced intrinsic metastasis potential in human breast cancer cells without increasing their transformation abilities. *Cancer Res.* 57:1199-1205 (1997).

Tandon, A.K., Clark, G.M., Chamness, G.C., Ullrich, A., and McGuire, W.L. HER-2/neu oncogene protein and prognosis in breast cancer. *J. Clin. Oncol.*, *7*: 1120-1128, 1989.

Tanguay, R.L. and Gallie, D.R. Translational efficiency is regulated by the length of the 3' untranslated region. *Mol. Cell. Biol.* **16**(1), 146-156 (1996).

Tyson, F.L., Boyer, C.M., Kaufman, R., O'Briant, K., Cram, G., Crews, J.R., Soper, J.T., Daly, L., Fowler, W.C., Jr., Haskill, J.S., and Bast, R.C., Jr. Expression and amplification of the HER-2/neu (c-erbB-2) protooncogene in epithelial ovarian tumors and cell lines. *American Journal of Obstetrics & Gynecology* **165**(3), 640-646 (1991).

Tzahar, E., & Yarden, Y. The ErbB-2/HER-2 oncogenic receptor of adenocarcinomas: from orphanhood to multiple stromal ligands. *Biochimica et Biophysicia Acta* **1377,** M25-M37 1998).

Tzahar E., Pinkas-Kramarski R., Moyer J.D., Klapper L.N., Alroy I., Levkowitz G., Shelly M., Henis S., Eisenstein M., Ratzkin B.J., Sela M., Andrews G.C., and Yarden Y. Bivalence of EGF-like ligands drives the ErbB signaling network. *EMBO J.* 16:4938-4950 (1997).

Tzahar E., Waterman H., Chen X., Levkowitz G., Karunagaran D., Lavi S., Ratzkin B.J., and Yarden Y. A hierarchical network of inter-receptor interactions determines signal transduction by NDF/neuregulin and EGF. *Mol. Cell. Biol.* 16:5276-5287 (1996).

Ullrich A. and Schlessinger J., Signal transduction by receptors with tyrosine kinase activity. *Cell* 61:203-212 (1990).

Ullrich, A., Coussens, L., Hayflick, J.S., Dull, T.J., Gray, A., Tam, A.W., Lee, J., Yarden, Y., Libermann, T.A., Schlessinger, J., Downward, J., Mayes, E.L.V., Whittle, N., Waterfield, M.D., and Seeburg, P. Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. *Nature* 309, 418-425 (1984).

van de Poll M.L.M., vann Vugt M.J.H., Lenferink A.E.G., and van Zoelen E.J.J. Insertions of Argos sequences into the B-loop of epiderman growth factor results in a low-affinity ligand with strong agonistic activity. Biochem. 36:7425-7431 (1997).

Van der Geer P., Hunter T., and Lindberg R.A. Receptor protein tyrosine kinases and their signal transduction pathways. Annu. Rev. Cell Biol. 10:251-337 (1994).

Vecchi, M., Baulida, J., and Carpenter, G. Selective cleavage of the heregulin receptor ErbB-4 by Protein kinase C activation. *J. Biol. Chem., 271*: 18989-18995, 1996.

Vecchi, M. and Carpenter, G. Constitutive proteolysis of the ErbB-4 receptor tyrosine kinase by a unique sequential mechanism. *J. Cell Biol., 139*: 995-1003, 1997.

Vogel, W., Gish, G.D., Alves, F., and Pawson, T. The discoidin domain receptor tyrosine kinases are activated by collagen. *Molec. Cell* 1, 13-23 (1997).

Vu, T.H., Martin, G.R., Lee, P., Mark, D., Wang, A., and Williams, L.T. Developmentally regulated use of alternative promoters creates a novel platelet-derived growth factor receptor transcript in mouse teratocarcinoma and embryonic stem cells. *Molec. Cell. Biol.* 9(10), 4563-4567 (1989).

Wallasch C., Weiss F.U. Niederfellner G., Jallal B., Issing W., and Ullrich A. Heregulin-dependent regulation of HER-2/neu oncogenic signaling by heterodimerization with HER-3. *EMBO J.* 14:4267-4275 (1995).

Weichen, K. and Dietel, M. c-erbB-2 anti-sense phosphorothioate oligodeoxynucleotides inhibit growth and serum-induced cell spreading of p185 c-erbB-2 —overexpressing ovarian carcinoma cells. *Int. J. Cancer* **63**, 604-608 (1995).

Weiner, D.B., Liu, J., Cohen, J.A., Williams, W.V., and Greene, M.I. A point mutation in the neu oncogene mimics ligand induction of receptor aggregation. *Nature* 339, 230-231 (1989).

Weiss, A. & Schlessinger, J. Switching signals on or off by receptor dimerization. *Cell* 94, 277-280 (1998).

Weiss F.Ul, Daub H., and Ullrich A. Novel mechanisms of RTK signal generation. *Curr. Opinion in Gen. and Dev.* 7:80-86 (1997).

Wen D., Peles E., Cupples R., Suggs S.V., Bacus S.S., Luo Y., Trail G., Hu S., Silbiger S.M., Ben Levy R., Koski R.A., Lu H.S. and Yarden Y. Neu differentiation factor: a transmembrane glycoprotein containing an EGF domain and an immunoglobulin homology unit. *Cell* 69:559-572 (1992).

Werner, S., Duan, D.-S.R., de Vries, C., Peters, K.G., Johnson, D.E., and Williams, L.T. Differential splicing in the extracellular region of fibroblast growth factor receptor 1 generates receptor variants with different ligand-binding specificities. *Molec. Cell. Biol.* 12(1), 82-88 (1992).

Wickens, M. Forward, backward, how much, when: mechanisms of poly(A) addition and removal and their role in early development. *Sem. Dev. Biol.* **3**, 399-412 (1992).

Wickens, M., Anderson, P., and Jackson, R.J. Life and death in the cytoplasm: messages from the 3' end. *Curr. Opin. Gene. Dev.* **7**, 220-232 (1997).

Woltjer R.L., Lukas T.J., and Staros J.V. Direct identification of residues of the epidermal growth factor receptor in close proximity to the amino terminus of bound epidermal growth factor. *Proc. Natl. Acad. Sci. USA* 89:7801-7805, (1992).

Xu, F.-J., Stack, S., Boyer, C., O'Briant, K., Whitaker, R., Mills, G.B., Yu, Y.H., and Bast, R.C., Jr. Heregulin and agonist anti-p185c-erbB2 antibodies inhibit proliferation but increase invasiveness of breast cancer cells that overexpress p185c-erbB2: Increased invasiveness may contribute to poor prognosis. *Clin. Cancer Res.* 3, 1629-1634 (1997).

Yamamoto, T., Ikawa, S., Akiyama, T., Semba, K., Nomura, N., Miyajima, N., Saito, T., and Toyoshima, K. Similarity of protein encoded by the human c-erb-B-2 gene to epidermal growth factor receptor. *Nature* **319**, 230-234 (1986).

Yamauchi, H.., O'Neill, A., Gelman, R., Carney, W., Tenney, D.Y., Hosch, S., and Hayes, D.F. Prediction of response to antiestrogen therapy in advanced breast cancer patients by pretreatment circulating levels of extracellular domain of the HER-2/c-neu protein. *J.Clin.Oncol., 15*: 2518-2525, 1997.

Yarden Y. and Ullrich A. Growth factor receptor tyrosine kinases. *Ann Rev. Biochem.* 57:443-478 (1988).

Yayon, A., Zimmer, Y., Guo-Hong, S., Avivi, A., Yarden, Y., and Givol, D. A confined variable region confers ligand specificity on fibroblast growth factor receptors: implications for the origin of the immunoglobulin fold. *EMBO J.* 11(5), 1885-1890 (1992).

Yu, D., Matin, A., Xia, W., Sorgi, F., Huang, L., and Hung, M.-C. Liposome-mediated *in vivo* E1A gene transfer suppressed dissemination of ovarian cancer cells that overexpress HER-2/*neu*. *Oncogene* **11**, 1383-1388 (1995).

Yu, D., Wolf, J.K., Scanlon, M., Price, J.E., and Hung, M.-C. Enhanced c-erbB-2/*neu* expression in human ovarian cancer cells correlates with more severe malignancy that can be suppressed by E1A. *Cancer Res.* **53**, 891-898 (1993).

Zabrecky, J.R., Lam, T., and McKenzie, S.J. The extracellular domain of p185/neu is released from the surface of human breast carcinoma cells, SK-BR-3. *J.Biol.Chem.* 266: 1716-1720, 1991.

Zhang, X., Silva, E., Gershenson, D., and Hung, M. Amplification and rearrangement of c-erbB proto-oncogenes in cancer of human genital tract. *Oncogene* **4**, 985-989 (1989).

Zhang D, Sliwkowski MX, Mark M, Frantz G, Akita R, Sun Y, Hillan K, Crowley C, Brush J, Godowski PJ. Neuregulin-3 (NRG3): a novel neural tissue-enriched protein that binds and activates ErbB4. *Proc Natl Acad Sci U S A* 94:9562-9567 (1997).

Zhang K., Sun J., Liu N., Wen D., Chang K., Thomason A., and Yoshinaga S.K. Transformation of NIH 3T3 cells by HER-3 or HER4 receptors requires the presence of HER 1 or HER2. *J. Biol. Chem.* 271:3884-3890 (1996).

Zhou, Q., Guo, Y., and Liu, Y. Regulation of the stability of heat-stable antigen mRNA by interplay between two novel *cis* elements in the 3' untranslated region. *Molec. Cell. Biol.* 18(2), 815-826 (1998).