CMLS, Cell. Mol. Life Sci. 61 (2004) 2965–2978
1420-682X/04/232965-14
DOI 10.1007/s00018-004-4277-7

CMLS Cellular and Molecular Life Sciences

Role of HER2/neu in tumor progression and therapy

S. Ménard *, P. Casalini, M. Campiglio, S. M. Pupa and E. Tagliabue

Molecular Targeting Unit, Department of Experimental Oncology, Istituto Nazionale Tumori, Via Venezian 1, 20133 Milano (Italy), Fax: +39 02 23903073, e-mail: sylvie.menard@istitutotumori.mi.it

Abstract. HER2 (human epidermal growth factor receptor-2; also known as erbB2) and its relatives HER1 (epidermal growth factor receptor; EGFR), HER3 and HER4 belong to the HER family of receptor tyrosine kinases. In normal cells, activation of this receptor tyrosine kinase family triggers a rich network of signaling pathways that control normal cell growth, differentiation, motility and adhesion in several cell lineages. The first tumor studied for an alteration of the HER2 oncogene is breast carcinoma, and so far the majority of studies have been performed on this oncotype. Although involvement of HER2 as a cause of human cell transformation needs to be further investigated, overexpression of the HER2 oncogene in human breast carcinomas has been associated with a more aggressive course of disease. It has been suggested that this association depends on HER2-driven proliferation, vessel formation and/or invasiveness; however, poor prognosis may not be directly related to the presence of the oncoprotein on the cell membrane but instead to the breast carcinoma subset identified by HER2 overexpression and characterized by a peculiar gene expression profile, as recently identified. HER2-positive tumors were recently shown to benefit from anthracyclin treatment and to be resistant to endocrine therapy. Despite the fact that many pathways interacting with HER2 are still not fully understood, this tyrosine kinase receptor is, to date, a promising molecule for targeted therapy.

Key words. HER2; biological role; prognostic value; predictive value; therapy.

Description of the normal counterpart and of its normal function

HER2 (human epidermal growth factor receptor-2; also known as erbB2) and its related HER1 (epidermal growth factor receptor; EGFR), HER3 and HER4 belong to the HER family of receptor tyrosine kinases. Individual features of this group of receptors include the ligand-less HER2 receptor and the kinase-dead HER3 receptors [1–4]. A large family of ligands (reviewed in [5]) induces receptor dimerization, with each ligand favoring some dimeric combinations over others in a specific hierarchical order, although a marked preference for HER2 as a dimer partner has been described [6, 7].

Expression patterns of HER receptors and their ligands, as well as targeted inactivation of components of the HER signaling network, have highlighted the importance of short-range ligand-receptor interactions, especially in mid-gestation processes. Apparently, the network is involved primarily in two types of interactions: (i) mesenchyme-epithelia cross-talk and (ii) neuronal effects on target cells, including muscle, astroglia, oligodendrocytes and Schwann cells. NRGs (neuregulins, a family of HER3 and HER4 ligands), for example, are synthesized by mesenchymal or neuronal cells and influence adjacent epithelial or non-neuronal cells, respectively, with respect to their differentiation, proliferation and migration. This may explain the crucial role of the HER receptor family in development of the cardiovascular system, nervous system, mammary gland and probably others.

An essential role for the HER receptor family in mid-gestation development was indicated by embryonic lethality of HER2- [8], HER4- [9] and NRG-deficient mice at around day 10 post-fertilization due to aberrant cardiac and peripheral nervous system development. The trabeculae, fingerlike extensions of the ventricular myocardium, fail to develop in these mice, and the resulting mutant heart is characterized by irregular beat, an enlarged common ventricle and reduced blood flow. HER3 knockout mice have less severe heart defects and consequently survive several days longer (to around embryonic day 13.5), displaying normal heart trabeculation but defective

* Corresponding author.

valve formation. The role of HER4 has been further established in HER4 knockout mice by re-expressing HER4 under the regulatory control of the cardiac α-myosin heavy chain (αMHC) promoter [10, 11]. Cardiac-rescued MHC-HER4 HER4$^{-/-}$ mice are viable but display abnormalities in the central nervous system and mammary gland. The mid-gestation cardiac defect in HER2 knockout animals has been circumvented through the use of a conditional mutagenesis approach. Conditional mutagenesis of the HER2 gene in murine ventricular cardiomyocytes [12] revealed development of severe dilated cardiomyopathy, with signs of cardiac dysfunction generally appearing by the second postnatal month. Based on these findings, the authors concluded that signaling from the HER2 receptor, which is enriched in T-tubules in cardiomyocytes, is crucial for adult heart function. In light of the adverse cardiac side effects observed in breast cancer patients treated with the monoclonal anti-HER2 antibody trastuzumab [13], an improved understanding of the molecular mechanisms by which HER2 regulates heart function is especially important.

NRGs exert many functions in neural development. Roles for NRG-1-HER signaling in neural development have been demonstrated in mice carrying an HER3-null mutation [14], as well as by selective mutations of NRG-1 ectodomains [15, 16], by MHC-HER-specific expression [10, 11] and by inducible HER2 ablation in Schwann cells [17]. In all of these mice, peripheral motorneuron axons defasciculate as they enter the muscle mass and fail to form mature neuromuscular junctions. Aberrant cranial nerve architecture and increased numbers of large interneurons within the cerebellum have been demonstrated in MHC-HER4 HER4$^{-/-}$ mice, while loss of NRG-1-HER signaling led to hypoplasia of the sympathetic ganglion chain and the neural crest-derived portion of the cranial sensory ganglia [14, 18–20]. Moreover, the NRG-1-HER mutants were completely devoid of Schwann cells in peripheral nerves at late development stages [15, 21]. Thus, NRG-1 signaling through the HER2/HER3 heterodimer is required for normal Schwann cell development. The role of HER2 signaling in later development of the Schwann cell lineage was also analyzed using conditional mutagenesis [17]. In that study, the conditional HER2 mutants displayed peripheral nerve hypomyelination associated with neuropathy, a phenotype reminiscent of the pathology in patients with Charcot-Marie-Tooth disease [22]. Thus HER2 is the first signaling molecule for which a role in control of Schwann cell myelination has been demonstrated in vivo. Moreover, Kim et al. have very recently demonstrated that HER2 signaling is also critical for oligodendrocyte differentiation in vivo [23]. To date however, no data have been reported on nervous system toxicity in patients treated with anti-HER2-targeted therapy. Nevertheless, patients treated long term should be carefully monitored for potential side effects, since a possible role for HER2 in mature nervous system tissue cannot be excluded.

In contrast to the embryonic lethality caused by HER2 inactivation, mice carrying a naturally occurring germ-line mutation in the kinase domain of EGFR known as Waved-2 (hypomorphic allele with severely reduced catalytic activity) are completely viable and display only epithelial defects, such as a wavy hair phenotype. Mutant mice display impaired epithelial development in several organs, resulting in phenotypes ranging from peri-implantation death to live progeny with abnormalities in multiple organs, such as liver and skin, depending on the genetic background [24, 25].

The importance of the HER receptor family and ligands in human mammary carcinoma has evoked keen interest in the normal functions of these receptors in the mammary gland, an organ that undergoes considerable postnatal development. Analyses of HER family ligands in mammary development have revealed a complicated picture. RNAs encoding the majority of the HER-specific growth factors such as AR (anphiregulin), BTC (betacellulin), HB-EGF (heparin-binding EGF), EPR (epiregulin), EGF (epidermal growth factor), NRG1 and TGFα (tumor growth factor α) are all present, each with a unique temporal pattern of transcriptional regulation during the normal course of mammary development, maturation and involution [26].

HERs play several normal non-oncogenic roles in regulating growth, differentiation, apoptosis and/or remodeling in normal mammary glands. These receptors are differentially expressed in mammary epithelial and/or stromal cells during various stages of development. In the mouse virgin gland, HER1 and HER2 colocalize in all major cell types during ductal morphogenesis but localize differentially in the mature gland. EGFR and HER2 are preferentially expressed in lactating ducts and alveoli, and HER3 and HER4 are more pronounced in alveoli [26]. Interestingly, a switch from HER3 to HER4 expression was observed in the developing mammary gland, suggesting that the two receptors play different roles in mammary morphogenesis. Activated EGFR and HER2 are highly expressed in extracts of mammary glands collected at puberty, suggesting a prominent role of these receptors at this stage of development, while both are expressed to only a minor extent in mammary glands in late-stage pregnancy and in lactation [27]. By contrast, HER3 and HER4 are active in mammary glands mostly during pregnancy and lactation [28]. With respect to the signaling pathways activated by HER2, Niemann et al., [29] demonstrated that formation of branched tubules relies on a pathway involving PI-3K, whereas alveolar morphogenesis requires MAPK.

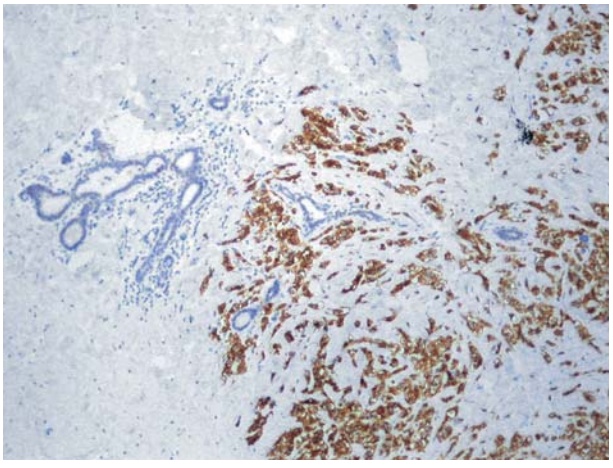
Mammary functions of HER2 have also been assessed using cytoplasmic, truncated dominant-negative form of the receptor, under the control of the mouse mammary

tumor virus (MMTV) promoter. Transgenic animals expressing dominant-negative MMTV-driven truncated HER2 have significant defects in mammary development late in gestation and early postpartum, with failure of alveolar expansion [30]. Ductal development occurs in these animals, but they have lactation problems, and mammary glands early postpartum show an immature phenotype more typical of late pregnancy [11]. These data do not reveal any indispensable role for a particular member of the family in mammary gland development. Moreover, the cross-talk between HERs and steroid hormone receptors in mammary gland development remains to be established. It seems very likely that these two receptor types act synergistically and that inhibition of both pathways is required for complete ablation of mammary gland development.

What are the types of cancer related to the described tyrosine kinase?

The first tumor studied for an alteration of the HER2 oncogene was breast carcinoma, and so far the majority of studies have been performed on this oncotype.

The literature reveals a wide variation in HER2 levels [31] within a single tumor type, most probably attributable to the lack of standardized methodologies used to assess HER2 status. The advent of standardized, FDA-approved tests for protein overexpression by immunohistochemistry (IHC) (Herceptest) (fig. 1a) or gene amplication by FISH (fig. 1b) has led to overall improvement in HER2 status assessment [32–34], as indicated by several quality control studies performed around the world [35, 36]. However, evaluation even with standardized IHC methodology has demonstrated wide variation in HER2 overexpression rates in different tumor types. The highest frequency of HER2 overexpression is found in inflammatory breast cancers scoring 3+ with Herceptest in more than 60% of the cases [37], followed by Wilm's tumor (50%), bladder cancer (44%) and non-inflammatory breast cancer (25%). In other tumors, including pancreatic, colon, ovary and lung tumors, the frequency of HER2 overexpression differs in different reports and is often greater than predicted by gene-amplification data [38–45], suggesting that overexpression in these tumors is due to gene deregulation rather than gene amplification. Breast carcinomas show the highest concordance between overexpression of the HER2 oncoprotein and amplification of the gene, making it possible to generate clinical data on the role of HER2 overexpression in disease progression.


A

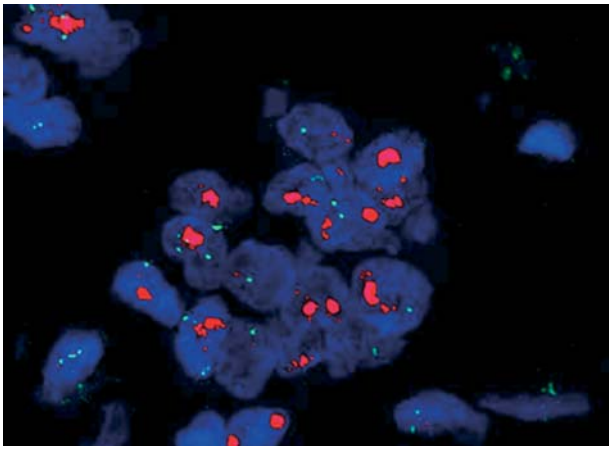

B

Figure 1. Evaluation of HER2 status in breast carcinomas. Paraffin-embedded sections were analyzed by immunohistochemistry (IHC) using Herceptest KIT supplies (DAKO) (*A*), or by fluorescence in situ hybridization (FISH) using HER2 Spectrum Orange-labeled probe, centromeric 17 Spectrum Green-labeled probe (Vysis) and DAPI as nucleus counterstaining (*B*)

What is known about cause/effect and the generation of certain tumors

Transgenic mice models have shown that the oncogenic rat HER2/neu, with an amino acid change in the extracellular domain is highly tumorigenic in mice with an early onset of mammary carcinomas in both females and males [46]. Also, the rat protooncogene HER2/neu induces mammary carcinomas, but the onset is late, variable and restricted to females [47]. In mice transgenic for this protooncogene, gene deletions involving removal of cysteine residues in the extracellular domain of the receptor were found to mediate transformation [48]. Similar deletion was not found in humans, where, however a splice variant of HER2 messenger RNA (mRNA), excluding the small exon 19 containing two cysteine residues was observed [48]. This physiologic splice variant of HER2 mRNA, whose role in human cell transformation is still not de-

fined, represents about 5% of all HER2 mRNA, and it has been shown to transform in vitro cells with high efficiency due to its constitutive activation occurring following spontaneous dimerization (fig. 2).

Despite the involvement of HER2 in cancer, its role in human cell transformation needs to be further investigated. Recent data have shown that HER2-positive breast carcinomas represent a particular tumor group with peculiar behavior. Various clinical studies have evaluated the relationship between HER2 and breast cancer outcome, and most have shown that women with HER2-positive tumors have a poorer prognosis than women with HER2-negative tumors [49–51]. However, while the prognostic value of HER2 amplification/overexpression in node-positive patients has been widely demonstrated, there is no consensus on its value in node-negative cases [52–55]. Although a few studies on small series have shown some prognostic impact of HER2 positivity in node-negative patients, others, including our study of a large cohort of node-negative cases, argue against a prognostic role for HER2 in this patient subset [56]. Moreover, in a recent microarray analysis to identify genes associated with poor prognosis (poor signature) and good prognosis (good signature) in node-negative patients, HER2 was not included in the 70 prognostic genes identified [57].

In any event, the prognostic impact of HER2 positivity is related only to the first 3–4 years after surgery, as indicated by the peak of early recurrences [56]. The reasons for early recurrences in HER2-positive tumors have been suggested to rest in events occurring at time of surgery. Indeed, growth factors released during wound healing [58, 59] have been shown to preferentially stimulate the growth of HER2-positive tumors [60, 61]. These growth factors are more likely to have a stimulatory effect in patients with disseminated micrometastasis (node-positive patients) of an HER2-positive tumor, which might also explain the prognostic impact of HER2 according to nodal status.

There is considerable interest in biologic markers able to predict the response of cancer patients to therapy. HER2 overexpression has been implicated as a potential indicator of responsiveness to doxorubicin. Indeed, the study by the Cancer and Acute Leukemia Group B (CALGB) of node-positive patients randomly allocated to three dose levels of CAF (cyclophosphamide, doxorubicin and fluorouracil) [62], as well as the study by Paik et al. [63] from the National Surgical Adjuvant Breast and Bowel Project (NSABP) examining the effect of doxorubicin in node-positive patients, and others [64] indicated that administration of doxorubicin was of significant benefit in HER2-positive tumors but without any beneficial effect in HER2-negative tumors.

Contrary to expectations based on most previous studies, HER2-positive tumors were recently shown to benefit from cyclophosphamide, methotrexate and fluorouracil (CMF) treatment [65, 66]; however, addition of doxorubicin to the CMF regimen further improved survival only in patients with HER2-positive tumors [67]. HER2 positivity has been related to endocrine therapy unresponsiveness, even in hormone receptor-positive patients [68, 69]. The recent observation that the level of expression of estrogen receptor is inversely correlated with HER2 expression [70], together with clinical data indicating that only high estrogen receptor-expressing tumors are sensitive to the anti-estrogen reagent tamoxifen [71], likely explain the tamoxifen resistance of HER2-positive tumors.

Is the decribed tyrosine kinase structurally altered or overexpressed in neoplastic cells? Molecular process for such alteration

In breast carcinomas, gene amplification is the key event for HER2 overexpression, whereas no mutation or genetic recombination has been described in human tumors, as opposed to experimental tumors in HER2/neu transgenic mice.

A new subset classification of breast carcinomas has recently been proposed based on the gene expression profile revealed by microarray analysis [72, 73]. HER2 overexpression, which is associated with overexpression of other genes that are probably co-amplified in the same amplicon, together with a series of other co-expressed genes, has been reported to identify a subset of tumors characterized by the lack of expression of genes associated with hormone receptor signaling pathways; high-level expression of a cluster of genes associated with proliferation; and expression of keratins associated with undifferentiated stem cells [74]. These findings suggest a pattern of gene expression peculiar to HER2-expressing tumors, and raise the possibility that all the clinical features thus far associated with HER2, e.g., prognosis, prediction of response to therapy, might actually be related to the biological behaviors of the subset and not directly to the presence of the oncoprotein. For example, sensitivity to anthracyclins might be related to the growth characteristics of the HER2-expressing subset rather than to the HER2 receptor pathway itself, as also suggested by in vitro studies [75].

What is known about the signal transduction pathways through which the tyrosine kinase exerts its oncogenic activity?

The diversification of HERs family during the evolution from one receptor/ligand in worm to four receptors/multiple ligands in mammals has created a complex network able to activate distinct signaling molecules downstream

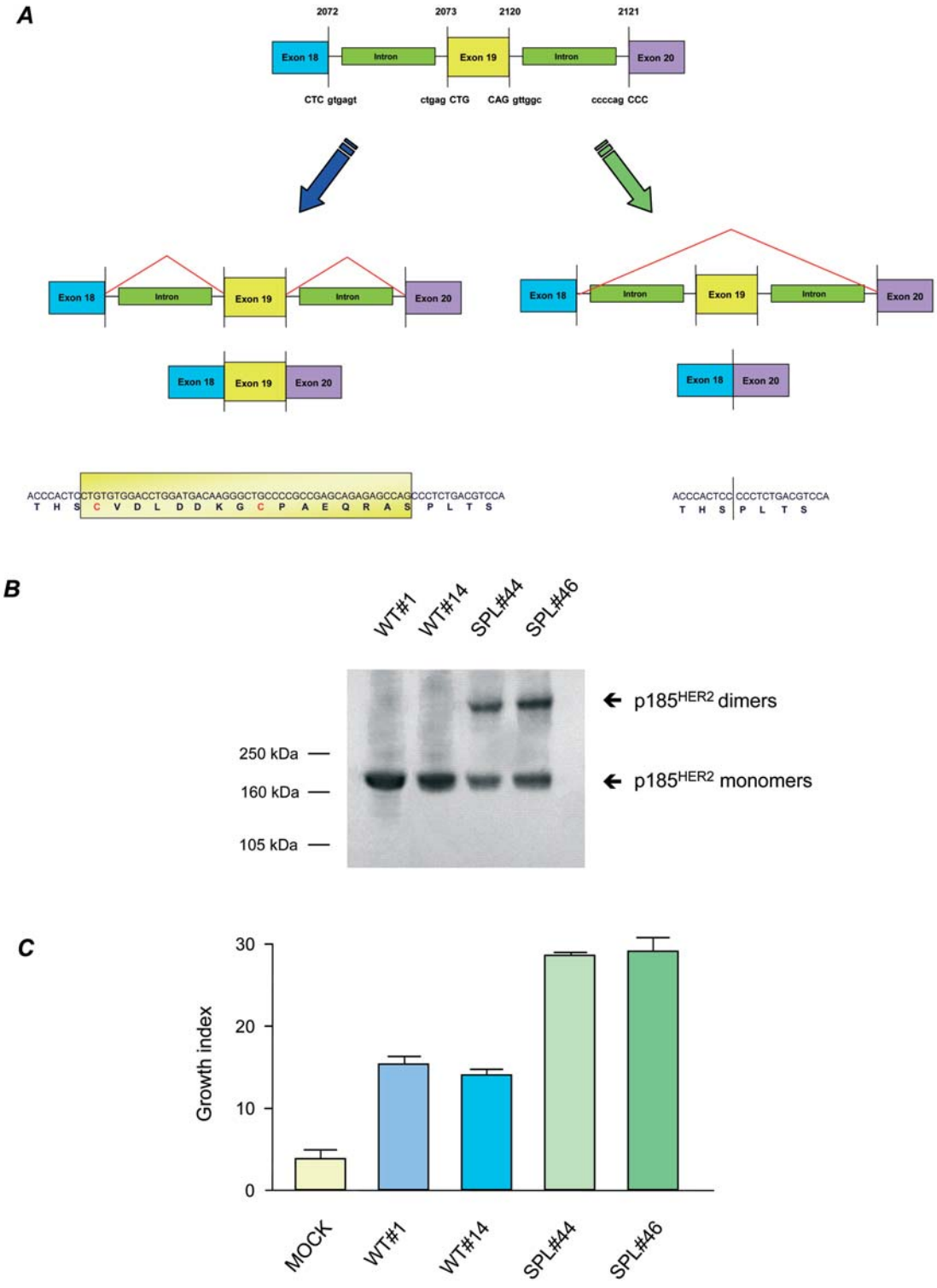


Figure 2. Schematic representation of the aberrant splicing event leading to the deletion of exon 19 (*A*). Western blot analysis of soluble extracts from NIH3T3 cells transfected with wild-type (WT#1 and 14) or splice (SPL#44 and 46) HER2 electrophoresed under non-reducing conditions, using monoclonal antibody against the intracellular domain of HER2 (*B*). Proliferation of the same transfected clones; MOCK represents NIH 3T3 cells transfected with empty vector (*C*).

of each receptor, providing a high degree of signaling diversity [76–78]. In normal tissue, the ligand-less HER2 functions only as a heterodimer with a ligand-bound receptor of the family, resulting the most prevalent dimers in several human tissues; HER2-containing heterodimer generates a stronger intracellular signal than that originating from other complexes (reviewed in [5]). In normal cells, activation of this receptor tyrosine kinase family triggers a rich network of signaling pathways that control normal cell growth, differentiation, motility and adhesion in several cell lineages [8]. The formation of heterodimers and the ensuing activation are temporary and spatially controlled in normal cells and tissue, but deregulation of this network has been reported in cancer cells, where increased expression levels of HER2 (e.g., in breast cancer) [79, 80] or HER1 (e.g., in lung cancer) [81] and the presence of an autocrine secretory loop of ligands or a paracrine growth induced by ligands secreted by adjacent stroma cells provide a growth advantage. Indeed, overexpression of HER2, which occurs in about 30% of breast and ovarian cancers [82], activates the PI3K/Akt 'survival' pathway, whose action favors cell proliferation apparently by inhibiting apoptosis [80, 83–85]. The 'survival' signal is normally coupled to the activation of the mitogenic signal involving MAPK-pathway recruitment. Both pathways mediate control on cell cycle progression by regulating effectors such as cyclin D1 and CDK inhibitors (p27 and p21) and by promoting ubiquitinilation of p53 [86–88]. Increased HER2 expression in cancer enhances and prolongs signaling from both the PI3K/Akt and MAPK pathways [89, 90] and induced deregulation of cell cycle checkpoints, associating upregulation of this receptor to the malignant phenotype. Hyperactivation of the PI3K/Akt pathway, observed in tumors upregulating HER2, and the genetic defect of molecules along apoptotic pathways prevent tumor cell apoptosis in these cancers. Recent proof that HER2 induction of proliferation in a normal mammary cellular context requires cooperation with survival-promoting factors supports the idea that the type of signal originating from overexpressed HER2 depends on additional defects in cell cycle control and suppressor gene activity [91]. In normal tissues, signaling by the HER family is controlled by several mechanisms that ensure the appropriate tuning of signals: dephosphorylation of receptors, dissociation of ligand receptor, downregulation of receptors by endocytosis that can target these molecules either to lysosomal degradation (promoting signal attenuation and decreas in mitogenicity) or receptor recycling to the plasma membrane (resulting in potentiation of receptor signaling). Hyperactivation of HER2 in cancer cells slowed down the major part of these processes (reviewed in [92]). New negative regulatory pathways of the HER family are now emerging, such as the receptor-associated late transducer (RALT) pathways, able to suppress the mitogenic and survival signal induced by HER receptors by directly interacting with all the different HER dimers with different relative potencies [93].

The tumorigenic action of HER2 is not limited to a potential proliferative effect. In fact, HER2 has been shown to be a metastasis-promoting factor. Changes in HER2 levels and in its activation by different EGF-like and heregulin (HRG) ligands have been associated with increased invasiveness in vitro and a more metastatic phenotype in vivo. A key role for HER family members in enhancing metastatic potential rests in their ability to promote secretion of basement membrane degradative enzymes, such as the matrix metalloproteases (MMPs) [94, 95], which determine modifications in the tissue architecture through the breakdown of the matrix and consequent perturbations of cell-cell and cell-matrix interactions. These alterations, together with changes in integrin and cadherin function frequently observed in tumor cells with activated HER, facilitate the communication between tumor cells and their escape from control by the microenvironment. In particular, the creation of new interactions of HER family members with integrins, such as HER2 and $\alpha_6\beta_4$ (fig. 3) and focal adhesion kinases recruit the PI3K pathway [96–98], inducing release of second messengers (phosphoinositides and calcium). These molecules in turn recruit multiprotein complexes that play a role in reorganization of actin-cytoskeleton and thus in tumor cell migration [99, 100]. Moreover, the high expression of HER2 in invadopodia of cancer cells suggests its involvement in cell migration [100, 101]. Although those interactions remain to be studied further,

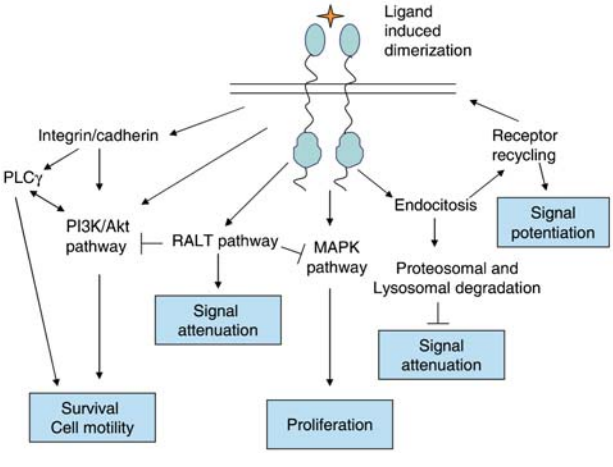


Figure 3. The major signaling pathways stimulated by activation of the HER family. Activation of receptor dimerization by ligand leads to activation of several pathways regulating proliferation, survival and cell motility. Several negative regulatory pathways counterbalance these effects by inducing signal attenuation, such as the pathway controlling HER-receptor degradation and the RALT pathways. Deregulation of HER receptors and their signaling in cancer can promote tumorigenesis.

the current body of information points to a predominant role for HER2 in motogenic and mitogenic signaling, whereas HER signaling appears to control the proteolysis of the matrix, mainly by inducing MMPs, and invasion [94, 99, 100]. By upregulating specific MMPs and angiogenic factors, such as VEGF [102], and by potentiating the adhesion of tumor cells to endothelial cells, activated HER members enhance angiogenesis and vascular invasion.

Position of the tyrosine kinase early or late

HER2 amplification and overexpression is an early event in breast cancerogenesis. Indeed, while it has never been found alterated in breast atypical hyperplasia, 'ductal carcinoma in situ' (DCIS), the pre-invasive lesion for breast cancer, is found to display HER2 overexpression in 50–60% of the cases (fig. 4), whereas the frequency decreases to 25% in invasive carcinomas. A possible explanation for this apparent discrepancy, considering a role of HER2 overexpression in increased invasiveness, is that DCIS is the pre-invasive lesion principally for HER2-positive invasive carcinomas, while the HER2-negative ones might derive directly from atypical hyperplasia.

Specific drugs available, description of results

Passive immunotherapy

Differential levels of HER2 expression in normal versus HER2-overexpressing tumor cells, together with the clear involvement of HER2 in tumor progression, make HER2 an ideal target for therapeutic approaches. Monoclonal antibodies specifically directed against the extracellular domain of HER2 have been shown to selectively inhibit growth of HER2-overexpressing cancer cells. One such antibody, 4D5 [103], recognizes an extracellular epitope in the cysteine-rich II domain residing very close to the transmembrane region [104]. To facilitate clinical use, 4D5 has been humanized at Genentech (South San Francisco, CA) by inserting its complementarity-determining regions (CDRs) into the human immunoglobulin G1 framework [105]. The therapeutic activity of the humanized antibody, known as trastuzumab, has been evaluated as a single agent given before [106] or after [107] traditional chemotherapy, and in combination with a variety of chemotherapy agents [13], in women with HER2-overexpressing metastatic breast cancer. The results indicated a therapeutic benefit of addition of trastuzumab to the therapeutic protocol. A recent study has also shown that preoperative trastuzumab in combination with other chemotherapeutic agents was active against HER2-overexpressing early-stage breast cancer [108]. Thus, trastuzumab has become a standard of care for women with HER2-overexpressing metastatic breast cancer, and its clinical efficacy seems to be clear. However, not all patients with HER2-overexpressing tumor found benefits by trastuzumab treatment, indicating the necessity to optimize the use of this therapeutic tool through the definition of its mechanism of action in vivo, which it is not yet completely understood. Analyses of trastuzumab activity in experimental models have evidenced at least three different possible mechanisms: HER2 downregulation, antibody-dependent cell cytotoxicity (ADCC) and alteration of vessel development. In vitro treatment of HER2-overexpressing breast carcinoma cell lines with trastuzumab resulted in downmodulation of the receptor and inhibition of tumor growth [109, 110]. Indeed, trastuzumab induces obligate formation of HER2 homodimers, leading to an increase of ligand-mediated endocytosis of the receptor and, consequently, to significant removal of HER2 from the plasma membrane and decreased receptor-initiated constitutive signaling [104, 109, 111]. Several other phenotypic changes accompany trastazumab binding to HER2-overexpressing cells in vitro, including induction of p27KIP1 and of the Rb-related protein, p130, with consequent reduction of the number of cells in S-phase [112]. In keeping, we also reported that ectopic expression of Rb2/p130 suppresses the tumorigenicity driven by HER2 overexpression in ovarian cancer cells [113]. To date, experiments evidencing a trastuzumab dose-dependent antitumor effect on human tumor cell lines xenografted in athymic mice have not included analysis of disappearance of the receptor [114]. In contrast, the activity of trastuzumab examined in animal models was found to depend on the engagement of Fc receptor-expressing lymphocytes [115], indicating ADCC as the major mechanism of antibody action. In FcR$\gamma^{+/+}$ nude mice injected subcutaneously with HER2-overexpressing human breast carcinoma cells, trastuzumab treatment re-

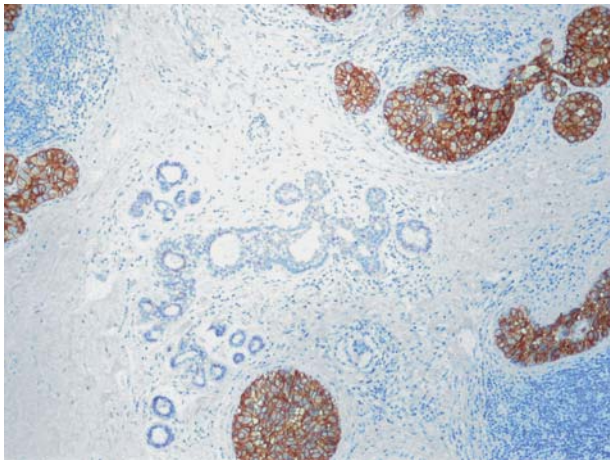


Figure 4. Overexpression of HER2 analyzed by immunohistochemistry (IHC) in ductal carcinoma in situ (DCIS).

sulted in near-complete (96%) inhibition of tumor growth. This protective effect was reduced more than 50% after disrupting the antibody's ability to engage cellular Fcγ receptor or in antibody-treated FcR$\gamma^{-/-}$ mice. A recent study in a preclinical model consisting of immunodeficient SCID mice transplanted with human breast carcinoma cells overexpressing HER2 showed that trastuzumab treatment inducing a 30% reduction of tumor volume was accompanied with a reduction in vessel volume, introducing a new mechanism of action for this antibody [116]. Indeed, HER2 signaling is known to control the expression of pro- and anti-angiogenic factors, including VEGF [102, 117], TGFα [118] and TSP-1 [116]. However, this finding does not necessarily exclude ADCC, since in a model such as the SCID mouse, which is profoundly deficient in T and NK cells, inhibition of HER2-driven pathways by the anti-HER2 antibody may contribute significantly to anti-tumor efficacy. The anti-tumor activity through vessel regression, together with the low protection exerted by trastuzumab found in mice knocked out for the Fc receptor, suggest that trastuzumab can utilize different mechanisms in vivo.

Clearly, the precise delineation of trastuzumab's mechanism of action in patients is essential for the design of more successful antibody treatment protocols in breast carcinoma patients. If, in fact, the major mechanism is ADCC, the combination of trastuzumab with immunosuppressive chemotherapeutic drugs requires optimal timing of trastuzumab delivery to enable rescue of ADCC effectors such as NK cells after chemotherapy. In that context, clinical studies have demonstrated a synergistic action between trastuzumab and the drug taxane [119], which induces suppression of adaptive immunity, but selectively increases NK activity [120]. To improve therapeutic activity, stimulation of NK activity might also be considered, utilizing cytokines or 'danger signals' such as unmethylated CpG-oligodeoxynucleotides, which enhance innate immunity [121].

Active immunotherapy

Cancer vaccination is based on the induction of long-lasting immunologic memory provided by expanded populations of T- and B-lymphocytes with cytotoxic potential and tumor antigen specificity.

Although HER2 is generally indicative of a poor prognosis, its overexpression is associated with a better outcome when inflammatory infiltrates are present in the tumor (fig. 5), suggesting a role for HER2 in tumor immunosurveillance [122–124]. In keeping, development of HER2-specific antibodies has been documented in some patients with primary HER2-positive tumors [125–127], and T-cells reactive to HER2 were found to occur naturally in patients with HER2-positive tumors [128], confirming the immunogenicity of the molecule.

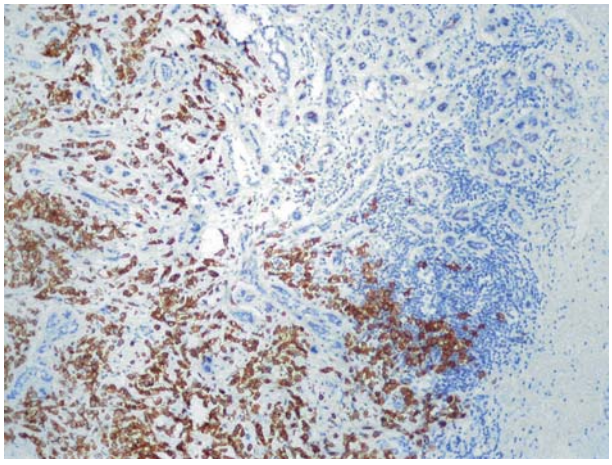


Figure 5. Presence of a marked inflammatory infiltrate in HER2-overexpressing infiltrating breast carcinoma. Paraffin-embedded tissue was probed with DAKO kit.

HER2-derived vaccines have been used in efforts to redirect immunity to induce rejection of HER2-positive tumors [129, 130]. Immunization regimens of active immunotherapy have been devised that generate specific T-cell responses with or without accompanying antibody responses and are currently being tested in animal models or in clinical trials [129–134].

Animal studies have shown that cancer vaccines generally display only limited or no efficacy in curing established tumors in syngeneic models [135]. Furthermore, the limited therapeutic efficacy of vaccines was lost when they were not given in the first few days after tumor cell implantation when tumor burden is still very small [136, 137]. These experimental findings point to the need for better selection of patients for enrollment in immunotherapy trials, such as patients with a minimal residual disease after conventional treatments or patients bearing tumors at an early stage of disease, to allow more reliable and objective clinical results [138–140].

Immunotherapeutic strategies such as immunization with MHC class I- and class II-restricted HER2-specific peptides with or without adjuvants, HER2 DNA, HER2 recombinant protein and dendritic cells loaded with HER2 peptides are now being tested in animal models and phase I clinical trials [129, 130, 141, 142]. In the majority of immunized patients, long-lasting T-cell-mediated immunity was revealed by in vitro and in vivo monitoring and, most importantly, immunity to the HER2 protein was significantly associated with epitope 'spreading', reflecting the initiation of an endogenous immune response [142].

In preclinical studies, the majority of cancer vaccines have been found to induce both T- and B-cell responses, even when no therapeutic benefits on experimental implanted tumors are observed.

While poorly active in inducing an anti-tumor therapeutic response, most of the vaccines are effective in preventing

spontaneous tumor occurrence [139]. Most data on the preventive potential of HER2 vaccines have come from studies in genetically predisposed animals transgenically expressing the rat HER2/neu proto-oncogene or its mutated activating form, and which are tolerant to self neu [143]. Different vaccination strategies include rat HER2/neu-positive allogeneic cells alone [144] or in combination with interleukin-12 [133]; the extracellular domain of the rat oncoprotein [145] or peptides derived from it [146]; and DNA plasmids encoding different fragments of HER2 or the full-length gene [131, 134, 147].

Immunological interventions targeting the HER2 protein, which is expressed at basal levels on adult normal tissues, have raised safety concerns with respect to the potential induction of autoimmune toxicity [148]; however, at present, no pertinent data in tumor-bearing hosts have been obtained [130, 149].

While therapeutic and prophylactic cancer vaccines may offer the advantage of inducing the development of both T- and B-cell-mediated long-term immunity, unlike the apparently short-lived therapeutic effects of passive therapies, an optimal vaccine formulation with objective therapeutic benefits remains to be defined. This goal awaits an improved understanding of the molecular mechanisms underlying the immunological escape of HER2-positive tumors.

Anti-receptor therapy

As previously reported, signals induced by HER2-containing heterodimers have the strongest biological activity with respect to the other HERs dimers [5], and high expression of HER2 in tumors leads to amplification of the signaling cascade induced by the EGF-family ligands [92, 150]. Since HER2 is also the preferred partner of HER1, inhibition of HER1 activity has been proposed as an avenue to block HER2 activity. The recent availability of tyrosine kinase inhibitors (TKIs) that specifically block the phosphorylation and function of HER1 has provided a powerful new tool for therapy of tumors whose proliferation depends on HER1 or HER2. One such inhibitor is ZD1839 (Iressa), an orally active, selective HER1 TKI that induces a reversible inhibition or a delay in the growth of cancer cell lines and human tumor xenografts expressing high levels of HER1 [151]. Following studies showed that tumors expressing low levels of HER1 or overexpressing HER2 in presence of normal/low HER1 are also sensitive to ZD1839 [152–155], whereas resistance to this TKI has been observed in cell lines with a high to low level of EGFR, suggesting that expression of the target receptor is not a marker of sensitivity to ZD1839 [154, 155]. In addition, treatment of breast carcinoma cell lines expressing both HER2 and hormone receptors with ZD1839 was found to restore the inhibitory activity of tamoxifen [156], suggesting the usefulness of TKI-mediated disruption of HER2 signaling in abrogating the anti-estrogen resistance observed in HER2-positive breast tumors. The precise mechanism through which Iressa exerts its antitumor effect remains unclear. The first step results in the inhibition of the EGFR tyrosine-kinase activity, leading to a blockage of the activation pathways that mediate EGFR functions. The failure of ZD1839 with conventional chemotherapeutic agents in phase III trials on unselected NSCLC have highlighted the lack of acknowledge of the ZD1839 mechanism of action as well as the absence of reliable markers of response to this drug (clinical endpoints). Elucidation of these points is mandatory to further design of new schedules of treatment with target-specific and conventional chemotherapies.

Despite the abundance of studies on the biology of HER2 and its receptor family, many pathways interacting with HER2 are still not fully understood or exploited for therapeutic purposes. However, HER2 is, to date, one of the most promising molecules for targeted therapy.

Acknowledgements. Partially supported by Associazione Italiana per la Ricerca sul Cancro and by Cariplo.

1 Plowman G. D., Green J. M., Culouscou J.-M., Carlton G. W., Rothwell V. M. and Buckley S. (1993) Heregulin induces tyrosine phosphorylation of HER4/p180erbB4. Nature **366:** 473–475
2 Coussens L., Yang-Feng T. L., Liao Y.-C., Chen E., Gray A., McGrath J. et al. (1985) Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. Science **230:** 1132–1139
3 Kraus M. H., Issing W., Miki T., Popescu N. C. and Aaronson S. A. (1989) Isolation and characterization of erbB3, a third member of the erbB/epidermal growth factor receptor family: evidence for overexpression in a subset of human mammary tumors. Proc. Natl. Acad. Sci. USA **86:** 9193–9197
4 Ullrich A., Coussens L., Hayflick J. S., Dull T. J., Gray A., Tam A. W. et al. (1984) Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. Nature **309:** 418–425
5 Yarden Y. (2001) The EGFR family and its ligands in human cancer. signalling mechanisms and therapeutic opportunities. Eur. J. Cancer **37:** S3–S8
6 Graus-Porta D., Beerli R. R., Daly J. M. and Hynes N. E. (1997) ErbB-2, the preferred heterodimerization partner of all ErbB receptor, is a mediator of lateral signaling. EMBO J. **16:** 1647–1655
7 Tzahar E., Waterman H., Chen X., Levkowitz G., Karunagaran D., Lavi S. et al. (1996) A hierarchinal network of interreceptor interactions determines signal transduction by neu differentiation factor/neuregulin and epidermal growth factor. Mol. Cell. Biol. **16:** 5276–5287
8 Lee K.-F., Simon H., Chen H., Bates B., Hung M.-C. and Hauser C. (1995) Requirement for neuregulin receptor erbB2 in neural and cardiac development. Nature **378:** 394–398
9 Gassmann M., Casagranda F., Orioli D., Simon H., Lai C., Klein R. et al. (1995) Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. Nature **378:** 390–394

10 Morris J. K., Lin W., Hauser C., Marchuk Y., Getman D. and Lee K. F. (1999) Rescue of the cardiac defect in ErbB2 mutant mice reveals essential roles of ErbB2 in peripheral nervous system development. Neuron **23:** 273–283

11 Tidcombe H., Jackson-Fisher A., Mathers K., Stern D. F., Gassmann M. and Golding J. P. (2003) Neural and mammary gland defects in ErbB4 knockout mice genetically rescued from embryonic lethality. Proc. Natl. Acad. Sci. USA **100:** 8281–8286

12 Ozcelik C., Erdmann B., Pilz B., Wettschureck N., Britsch S., Hubner N. et al. (2002) Conditional mutation of the ErbB2 (HER2) receptor in cardiomyocytes leads to dilated cardiomyopathy. Proc. Natl. Acad. Sci. USA **99:** 8880–8885

13 Slamon D. J., Leyland-Jones B., Shak S., Fuchs H., Paton V., Bajamonde A. et al. (2001) Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N. Engl. J. Med. **344:** 783–792

14 Riethmacher D., Sonnenberg-Riethmacher E., Brinkmann V., Yamaai T., Lewin G. R. and Birchmeier C. (1997) Severe neuropathies in mice with targeted mutations in the ErbB3 receptor. Nature **389:** 725–730

15 Wolpowitz D., Mason T. B., Dietrich P., Mendelsohn M., Talmage D. A. and Role L. W. (2000) Cysteine-rich domain isoforms of the neuregulin-1 gene are required for maintenance of peripheral synapses. Neuron **25:** 79–91

16 Meyer D., Yamaai T., Garratt A., Riethmacher-Sonnenberg E., Kane D., Theill L. E. et al. (1997) Isoform-specific expression and function of neuregulin. Development **124:** 3575–3586

17 Garratt A. N., Voiculescu O., Topilko P., Charnay P. and Birchmeier C. (2000) A dual role of erbB2 in myelination and in expansion of the schwann cell precursor pool [In Process Citation]. J. Cell Biol. **148:** 1035–1046

18 Britsch S., Li L., Kirchhoff S., Theuring F., Brinkmann V., Birchmeier C. and Riethmacher D. (1998) The ErbB2 and ErbB3 receptors and their ligand, neuregulin-1, are essential for development of the sympathetic nervous system. Genes Dev. **12:** 1825–1836

19 Erickson S. L., O'Shea K. S., Ghaboosi N., Loverro L., Frantz G., Bauer M. et al. (1997) ErbB3 is required for normal cerebellar and cardiac development: a comparison with ErbB2- and heregulin-deficient mice. Development **124:** 4999–5011

20 Kramer R., Bucay N., Kane D. J., Martin L. E., Tarpley J. E. and Theill L. E. (1996) Neuregulins with an Ig-like domain are essential for mouse myocardial and neuronal development. Proc. Natl. Acad. Sci. USA **93:** 4833–4838

21 Woldeyesus M. T., Britsch S., Riethmacher D., Xu L., Sonnenberg-Riethmacher E., Abou-Rebyeh F. et al. (1999) Peripheral nervous system defects in erbB2 mutants following genetic rescue of heart development. Genes Dev. **13:** 2538–2548

22 Benstead T. J. and Grant I. A. (2001) Progress in clinical neurosciences: Charcot-Marie-Tooth disease and related inherited peripheral neuropathies. Can. J. Neurol. Sci. *2***8:** 199–214

23 Kim J. Y., Sun Q., Oglesbee M. and Yoon S. O. (2003) The role of ErbB2 signaling in the onset of terminal differentiation of oligodendrocytes in vivo. J. Neurosci. **23:** 5561–5571

24 Luetteke N. C., Phillips H. K., Qiu T. H., Copeland N. G., Earp H. S., Jenkins N. A. et al. (1994) The mouse waved-2 phenotype results from a point mutation in the EGF receptor tyrosine kinase. Genes Dev. **8:** 399–413

25 Fowler K. J., Walker F., Alexander W., Hibbs M. L., Nice E. C., Bohmer R. M. et al. (1995) A mutation in the epidermal growth factor receptor in waved-2 mice has a profound effect on receptor biochemistry that results in impaired lactation. Proc. Natl. Acad. Sci. USA **92:** 1465–1469

26 Schroeder J. A. and Lee D. C. (1998) Dynamic expression and activation of ERBB receptors in the developing mouse mammary gland. Cell Growth Differ. **9:** 451–464

27 Sebastian J., Richards R. G., Walker M. P., Wiesen J. F., Werb Z., Derynck R. et al.. (1998) Activation and function of the epidermal growth factor receptor and erbB-2 during mammary gland morphogenesis. Cell Growth Differ. **9:** 777–785

28 Yang Y., Spitzer E., Meyer D., Sachs M., Niemann C., Hartmann G. et al. (1995) Sequential requirement of hepatocyte growth factor and neuregulin in the morphogenesis and differentiation of the mammary gland. J. Cell Biol. **131:** 215–226

29 Niemann C., Brinkmann V., Spitzer E., Hartmann G., Sachs M., Naundorf H. et al. (1998) Reconstitution of mammary gland development In vitro: requirement of c- met and c-erbB2 signaling for branching and alveolar morphogenesis [In Process Citation]. J. Cell Biol. **143:** 533–545

30 Jones F. E. and Stern D. F. (1999) Expression of dominant-negative ErbB2 in the mammary gland of transgenic mice reveals a role in lobuloalveolar development and lactation. Oncogene **18:** 3481–3490

31 Ménard S., Casalini P., Campiglio M., Pupa S. M., Agresti R. and Tagliabue E. (2001) HER2 overexpression in various tumor types, focusing on its relationship to the development of invasive breast cancer. Ann. Oncol. **12:** S15–S19

32 Gancberg D., Jarvinen T., Di Leo A., Rouas G., Cardoso F., Paesmans M. et al. (2002) Evaluation of HER2/NEU protein expression in breast cancer by immunohistochemistry: an interlaboratory study assessing the reproducibility of HER2/NEU testing. Breast Cancer Res. Treat. **74:** 113–120

33 Rhodes A., Jasani B., Anderson E., Dodson A. R. and Balaton A. J. (2002) Evaluation of HER2/neu immunohistochemical assay sensitivity and scoring on formalin-fixed and paraffin-processed cell lines and breast tumors: a comparative study involving results from laboratories in 21 countries. Am. J. Clin. Pathol. **118:** 408–417

34 Paik S., Bryant J., Tan-Chiu E., Romond E., Hiller W., Park K. et al. (2002) Real-world performance of HER2 testing-national surgical adjuvant breast and bowel project experience. J. Natl. Cancer Inst. **94:** 852–854

35 Cell Markers and Cytogenetics Committees College of American Pathologists (2002) Clinical laboratory assays for HER2/neu amplification and overexpression: quality assurance, standardization, and proficiency testing. Arch. Pathol. Lab. Med. **126:** 803–808

36 Roche P. C., Suman V. J., Jenkins R. B., Davidson N. E., Martino S., Kaufman P. A. et al. (2002) Concordance between local and central laboratory HER2 testing in the breast intergroup trial N9831. J. Natl. Cancer Inst. **94:** 855–857

37 Tagliabue E., Pilotti S., Gianni A. M., Ménard S. and Colnaghi M. I. (1998) Target molecules for immunotherapy of inflammatory breast carcinomas. Eur. J. Cancer **34:** 1982–1983

38 Nakamura H., Saji H., Ogata A., Hosaka M., Hagiwara M., Kawasaki N. et al. (2003) Correlation between encoded protein overexpression and copy number of the HER2 gene with survival in non-small cell lung cancer. Int. J. Cancer **103:** 61–66

39 Goebel S. U., Iwamoto M., Raffeld M., Gibril F., Hou W., Serrano J. et al. (2002) Her-2/neu expression and gene amplification in gastrinomas: correlations with tumor biology, growth and aggressiveness. Cancer Res. **62:** 3702–3710

40 Yan J., Fang Y., Huang B. J., Liang Q. W., Wu Q. L. and Zeng Y. X. (2002) Absence of evidence for HER2 amplification in nasopharyngeal carcinoma. Cancer Genet. Cytogenet. **132:** 116–119

41 Latif Z., Watters A. D., Bartlett J. M., Underwood M. A. and Aitchison M. (2002) Gene amplification and overexpression of HER2 in renal cell carcinoma. BJU Int. **89:** 5–9

42 Khan A. J., King B. L., Smith B. D., Smith G. L., DiGiovanna M. P., Carter D. et al. (2002) Characterization of the HER2/neu oncogene by immunohistochemical and fluorescence in situ hybridization analysis in oral and oropharyngeal squamous cell carcinoma. Clin. Cancer Res. **8:** 540–548

43 Soule S., Baldridge L., Kirkpatrick K., Cheng L., Gilbert J. L., Smith L. R. et al. (2002) HER2/neu expression in germ cell tumours. J. Clin. Pathol. **55:** 656–658

44 Geddert H., Zeriouh M., Wolter M., Heise J. W., Gabbert H. E. and Sarbia M. (2002) Gene amplification and protein overexpression of c-erb-b2 in Barrett carcinoma and its precursor lesions. Am. J. Clin. Pathol. **118:** 60–66

45 Hirsch F. R., Varella-Garcia M., Franklin W. A., Veve R., Chen L., Helfrich B. et al. (2002) Evaluation of HER2/neu gene amplification and protein expression in non-small cell lung carcinomas. Br. J. Cancer **86:** 1449–1456

46 Siegel P. M. and Muller W. J. (1996) Mutations affecting conserved cysteine residues within the extracellular domain of Neu promote receptor dimerization and activation. Proc. Natl. Acad. Sci USA **93:** 8878–8883

47 Guy C. T., Webster M. A., Schaller M., Parsons T. J., Cardiff R. D. and Muller W. J. (1992) Expression of the *neu* protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. Proc. Natl. Acad. Sci. USA **89:** 10578–10582

48 Siegel P. M., Ryan E. D., Cardiff R. D. and Muller W. J. (1999) Elevated expression of activated forms of Neu/ErbB-2 and Erb–3 are involved in the induction of mammary tumors in transgenic mice: implicatins for human breast cancer. EMBO J. **18:** 2149–2164

49 Ménard S., Forti S., Castiglioni F., Agresti R. and Balsari A. (2001) HER2 as a prognostic factor in breast cancer. Oncology **61:** 67–72

50 Masood S. and Bui M. M. (2002) Prognostic and predictive value of HER2/neu oncogene in breast cancer. Microsc. Res. Tech. **59:** 102–108

51 Ménard S., Tagliabue E., Campiglio M. and Pupa S. M. (2000) Role of HER2 gene overexpression in breast carcinoma. J. Cell Physiol. **182:** 150–162

52 Press M. F., Bernstein L., Thomas P. A., Meisner L. F., Zhou J. Y., Ma Y. L. et al. (1997) HER2/*neu* gene amplification characterized by fluorescence in situ hybridization: poor prognosis in node-negative breast carcinomas. J. Clin. Oncol. **15:** 2894–2904

53 Cooke T., Reeves J., Lannigan A. and Stanton P. (2001) The value of the human epidermal growth factor receptor-2 (HER2) as a prognostic marker. Eur. J. Cancer **37:** 3–10

54 Volpi A., De Paola F., Nanni O., Granato A. M., Bajorko P., Becciolini A. et al. (2000) Prognostic significance of biologic markers in node-negative breast cancer patients: a prospective study. Breast Cancer Res. Treat. **63:** 181–192

55 Ross J. S., Muraca P. J. and Jaffe D. (1998) Multivariate analysis of prognostic factors in lymph node negative breast cancer. Mod. Pathol. **11:** 26a

56 Ménard S., Balsari A., Casalini P., Tagliabue E., Campiglio M., Bufalino R. et al. (2002) HER2-positive breast carcinomas as a particular subset with peculiar clinical behaviors. Clin. Cancer Res. **8:** 520–525

57 Van'T Veer L. J., Dai H., Van de Vijver M. J., He Y. D., Hart A. A., Mao M. et al. (2002) Gene expression profiling predicts clinical outcome of breast cancer. Nature **415:** 530–536

58 Murthy S. M., Goldschmidt R. A., Rao L. N., Ammirati M., Buchmann T. and Scanlon E. F. (1989) The influence of surgical trauma on experimental metastasis. Cancer **64:** 2035–2044

59 Sieweke M. H. and Bissell M. J. (1994) The tumor-promoting effect of wounding: a possible role for TGF-beta-induced stromal alterations. Crit Rev. Oncog. **5:** 297–311

60 Tagliabue E., Agresti R., Ghirelli C., Morelli D. and Ménard S. (2001) Letter to the editor. The early relapse of premenopausal patients after surgery for node-positive breast carcinoma. Breast Cancer Res. Treat. **70:** 155–156

61 Tagliabue E., Agresti R., Carcangiu M. L., Ghirelli C., Morelli D., Campiglio M. et al. (2003) Role of HER2 in wound-induced breast carcinoma proliferation. Lancet **362:** 527–533

62 Thor A. D., Berry D. A., Budman D. R., Muss H. B., Kute T., Henderson I. C. et al. (1998) erbB-2, p53 and efficacy of adjuvant therapy in lymph node- positive breast cancer. J. Natl. Cancer Inst. **90:** 1346–1360

63 Paik S. M., Bryant J., Park C. H., Fisher B., Tan-Chiu E., Hyams D. et al. (1998) erbB-2 and response to doxorubicin in patients with axillary lymph node-positive, hormone receptor-negative breast cancer. J. Natl. Cancer Inst. **90:** 1361–1370

64 Di Leo A., Gancberg D., Larsimont D., Tanner M., Jarvinen T., Rouas G. et al. (2002) HER2 amplification and topoisomerase IIalpha gene aberrations as predictive markers in node-positive breast cancer patients randomly treated either with an anthracycline-based therapy or with cyclophosphamide, methotrexate, and 5-fluorouracil. Clin. Cancer Res. **8:** 1107–1116

65 Ozinsky A., Underhill D. M., Fontenot J. D., Hajjar A. M., Smith K. D., Wilson C. B. et al. (2000) The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. Proc. Natl. Acad. Sci. USA **97:** 13766–13771

66 Ménard S., Valagussa P., Pilotti S., Gianni L., Biganzoli E., Boracchi P. et al. (2001) Response to Cyclophosphamide, Methotrexate, and Fluorouracil in lymph node-positive breast cancer according to HER2 overexpression and other tumor biologic variables. J. Clin. Oncol. **19:** 329–335

67 Moliterni A., Ménard S., Valagussa P., Biganzoli E., Boracchi P., Balsari A. et al. (2003) HER2 overexpression and doxorubicin in the adjuvant chemotherapy of resectable breast cancer. J. Clin. Oncol. **21:** 458–462

68 Grunt T. W., Saceda M., Martin M. B., Lupu R., Dittrich E., Krupitza G. et al. (1995) Bidirectional interactions between the estrogen receptor and the c-erbB-2 signaling pathways: Heregulin inhibits estrogenic effects in breast cancer cells. Int. J. Cancer **63:** 560–567

69 Elledge R. M., Green S., Ciocca D., Pugh R., Allred D. C., Clark G. M. et al. (1998) HER2 expression and response to tamoxifen in estrogen receptor-positive breast cancer: a southwest oncologty group study. Clin. Cancer Res. **4:** 7–12

70 Konecny G., Pauletti G., Pegram M., Untch M., Dandekar S., Aguilar Z. et al. (2003) Quantitative association between HER2/neu and steroid hormone receptors in hormone receptor-positive primary breast cancer. J. Natl. Cancer Inst. **95:** 142–153

71 Early Breast Cancer Trialists' Collaborative Group (1998) Tamoxifen for early breast cancer: an overview of the randomised trials. Lancet **351:** 1451–1467

72 Perou C. M., Sorlie T., Eisen M. B., van de Rijn M., Jeffrey S. S., Rees C. A. et al. (2000) Molecular portraits of human breast tumours. Nature **406:** 747–752

73 Sorlie T., Perou C. M., Tibshirani R., Aas T., Geisler S., Johnsen H. et al. (2001) Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc. Natl. Acad. Sci. USA **98:** 10869–10874

74 Bocker W., Moll R., Poremba C., Holland R., Van Diest P. J., Dervan P. et al. (2002) Common adult stem cells in the human breast give rise to glandular and myoepithelial cell lineages: a new cell biological concept. Lab. Invest. **82:** 737–746

75 Campiglio M., Somenzi G., Olgiati C., Beretta G., Balsari A., Zaffaroni N. et al. (2003) Role of proliferation in HER2 status predicted response to doxorubicin. Int. J. Cancer **105:** 568–573

76 Aroian R. V., Koga M., Mendel J. E., Ohshima Y. and Sternberg P. W. (1990) The *let-23* gene necessary for *Caenorhabditis elegans* vulval induction encodes a tyrosine kinase of the EGF receptor subfamily. Nature **348:** 693–699

77 Wasserman J. D. and Freeman M. (1998) An autoregulatory cascade of EGF receptor signaling patterns the *Drosophila* egg. Cell **95:** 355–364

78 Stein R. A. and Staros J. V. (2000) Evolutionary analysis of the ErbB receptor and ligand families. J Mol. Evol. **50:** 397–412
79 Pierce J. H., Arnstein P., DiMarco E., Artrip J., Kraus M. H., Lonardo F. et al. (1991) Oncogenic potential of *erb*B-2 in human mammary epithelial cells. Oncogene **6:** 1189–1194
80 Liu Y., El-Ashry D., Chen D., Ding I. Y. F. and Kern F. G. (1995) MCF–7 breast cancer cells overexpressing transfected *c- erb*B-2 have an in vitro growth advantage in estrogen-depleted conditions and reduced estrogen-dependence and tamoxifen-sensitivity in vivo. Breast Cancer Res. Treat. **34:** 97–117
81 Franklin W. A., Veve R., Hirsch F. R., Helfrich B. A. and Bunn P. A. Jr (2002) Epidermal growth factor receptor family in lung cancer and premalignancy. Semin. Oncol. **29:** 3–14
82 Slamon D. J., Godolphin W., Jones L. A., Holt J. A., Wong S. C., Keith D. E. et al. (1989) Studies of the HER2/neu proto-oncogene in human breast and ovarian cancer. Science **244:** 707–712
83 Zhou B. P. and Hung M. C. (2002) Novel targets of Akt, p21(Cipl/WAF1), and MDM2. Semin. Oncol. **29:** 62–70
84 Di Fiore P. P., Pierce J. H., Kraus M. H., Segatto O., King C. R. and Aaronson S. A. (1987) erbB-2 is a potent oncogene when overexpressed in NIH/3T3 cells. Science **237:** 178–182
85 Muthuswamy S. K., Li D., Lelievre S., Bissell M. J. and Brugge J. S. (2001) ErbB2, but not ErbB1, reinitiates proliferation and induces luminal repopulation in epithelial acini. Nat. Cell Biol. **3:** 785–792
86 Lane H. A., Beuvink I., Motoyama A. B., Daly J. M., Neve R. M. and Hynes N. E. (2000) ErbB2 potentiates breast tumor proliferation through modulation of p27(Kip1)-Cdk2 complex formation: receptor overexpression does not determine growth dependency. Mol. Cell Biol. **20:** 3210–3223
87 Zhou B. P., Liao Y., Xia W., Spohn B., Lee M. H. and Hung M. C. (2001) Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER2/neu-overexpressing cells. Nat. Cell Biol. **3:** 245–252
88 Zhou B. P., Liao Y., Xia W., Zou Y., Spohn B. and Hung M. C. (2001) HER2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation. Nat. Cell Biol. **3:** 973–982
89 Karunagaran D., Tzahar E., Beerli R. R., Chen X. M., Graus-Porta D., Ratzkin B. J. et al. (1996) ErbB-2 is a common auxiliary subunit of NDF and EGF receptors: implications for breast cancer. EMBO J. **15:** 254–264
90 Waterman H., Alroy I., Strano S., Seger R. and Yarden Y. (1999) The C-terminus of the kinase-defective neuregulin receptor ErbB–3 confers mitogenic superiority and dictates endocytic routing. EMBO J. **18:** 3348–3358
91 Debnath J., Mills K. R., Collins N. L., Reginato M. J., Muthuswamy S. K. and Brugge J. S. (2002) The role of apoptosis in creating and maintaining luminal space within normal and oncogene-expressing mammary acini. Cell **111:** 29–40
92 Harari D. and Yarden Y. (2000) Molecular mechanisms underlying ErbB2/HER2 action in breast cancer. Oncogene **19:** 6102–6114
93 Anastasi S., Fiorentino L., Fiorini M., Fraioli R., Sala G., Castellani L. et al. (2003) Feedback inhibition by RALT controls signal output by the ErbB network. Oncogene **22:** 4221–4234
94 O-charoenrat P., Rhys-Evans P., Court W. J., Box G. M. and Eccles S. A. (1999) Differential modulation of proliferation, matrix metalloproteinase expression and invasion of human head and neck squamous carcinoma cells by c-erbB ligands. Clin. Exp. Met. **17:** 631–639
95 Tan M., Yao J. and Yu D. H. (1997) Overexpression of the c-*erb*B-2 gene enhanced intrinsic metastasis potential in human breast cancer cells without increasing their transformation abilities. Cancer Res. **57:** 1199–1205
96 Campiglio M., Tagliabue E., Uppugunduri S., Pellegrini R., Martignone S., Ménard S. et al. (1994) Colocalization of the p185^{HER2} oncoprotein and integrin $\alpha 6\beta 4$ in Calu-3 lung carcinoma cells. J. Cell. Biochem. **55:** 409–418
97 Tagliabue E., Ardini E., Pellegrini R., Campiglio M., Bufalino R., Jeschke M. et al. (1996) Laminin activates the p185HER2 oncoprotein and mediates growth inhibition of breast carcinoma cells. Br. J. Cancer **74:** 1427–1433
98 Gambaletta D., Marchetti A., Benedetti L., Mercurio A. M., Sacchi A. and Falcioni R. (2000) Cooperative signaling between alpha(6)beta(4) integrin and ErbB-2 receptor is required to promote phosphatidylinositol 3-kinase-dependent invasion. J. Biol. Chem. **275:** 10604–10610
99 Feldner J. C. and Brandt B. H. (2002) Cancer cell motility – on the road from c-erbB-2 receptor steered signaling to actin reorganization. Exp. Cell Res. **272:** 93–108
100 Dittmar T., Husemann A., Schewe Y., Nofer J. R., Niggemann B., Zanker K. S. et al. (2002) Induction of cancer cell migration by epidermal growth factor is initiated by specific phosphorylation of tyrosine 1248 of c-erbB-2 receptor via EGFR. FASEB J. **16:** 1823–1825
101 Eccles S. A. (2000) Cell biology of lymphatic metastasis. The potential role of c-erbB oncogene signalling. Recent Results Cancer Res. **157:** 41–54
102 Kerbel R. S., Viloria-Petit A., Klement G. and Rak J. (2000) 'Accidental' anti-angiogenic drugs. Anti-oncogene directed signal transduction inhibitors and conventional chemotherapeutic agents as examples. Eur. J. Cancer **36:** 1248–1257
103 Fendly B. M., Winget M., Hudziak R. M., Lipari M. T., Napier M. A. and Ullrich A. (1990) Characterization of murine monoclonal antibodies reactive to either the human epidermal growth factor receptor or HER2/neu gene product. Cancer Res. **50:** 1550–1558
104 De Santes K., Slamon D., Anderson S. K., Shepard M., Fendly B., Maneval D. et al. (1992) Radiolabeled antibody targeting of the HER2/neu oncoprotein. Cancer Res. **52:** 1916–1923
105 Carter P., Presta L., Gorman C. M., Ridgway J. B., Henner D., Wong W. L. et al. (1992) Humanization of an anti-p185^{HER2} antibody for human cancer therapy. Proc. Natl. Acad. Sci. USA **89:** 4285–4289
106 Vogel C. L., Cobleigh M. A., Tripathy D., Gutheil J. C., Harris L. N., Fehrenbacher L. et al. (2002) Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. J. Clin. Oncol. **20:** 719–726
107 Cobleigh M. A., Vogel C. L., Tripathy D., Robert N. J., Scholl S., Fehrenbacher L. et al. (1999) Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. J. Clin. Oncol. **17:** 2639–2648
108 Burstein H. J., Harris L. N., Gelman R., Lester S. C., Nunes R. A., Kaelin C. M. et al. (2003) Preoperative therapy with trastuzumab and paclitaxel followed by sequential adjuvant doxorubicin/cyclophosphamide for HER2 overexpressing stage II or III breast cancer: a pilot study. J. Clin. Oncol. **21:** 46–53
109 Hudziak R. M., Lewis G. D., Winget M., Fendly B. M., Shepard H. M. and Ullrich A. (1989) p185HER2 monoclonal antibody has antiproliferative effects in vitro and sensitizes human breast tumor cells to tumor necrosis factor. Mol. Cell. Biol. **9:** 1165–1172
110 Hieken T. J., Mehta R. R. and Shilkaitis A. (1996) HER2/neu and p53 expression in breast cancer: valid prognostic markers when assessed by direct immunoassay, but not by immunohistochemistry. Proc. Annu. Meet. Am. Soc. Clin. Oncol. **15:** 113
111 Sarup J. C., Johnson R. M., King K. L., Fendly B. M., Lipari M. T., Napier M. A. et al. (1991) Characterization of an anti-p185HER2 monoclonal antibody that stimulates receptor function and inhibits tumor cell growth. Growth Regul. **1:** 72–82

112 Sliwkowski M. X., Lofgren J. A., Lewis G. D., Hotaling T. E., Fendly B. M. and Fox J. A. (1999) Nonclinical studies addressing the mechanism of action of trastuzumab (Herceptin). Semin. Oncol. **26:** 60–70

113 Pupa S. M., Howard C. M., Invernizzi A. M., De Vecchi R., Giani C., Claudio P. P. et al. (1999) Ectopic expression of pRB2/p130 suppresses the tumorigenicity of c-erbB-2-overexpressing SkOv3 tumor cell line. Oncogene **18:** 651–656

114 Shepard H. M., Lewis G. D., Sarup J. C., Fendly B. M., Maneval D., Mordenti J. et al. (1991) Monoclonal antibody therapy of human cancer: taking the HER2 protooncogene to the clinic. J. Clin. Immunol. **11:** 117–127

115 Clynes R. A., Towers T. L., Presta L. G. and Ravetch J. V. (2000) Inhibitory Fc receptors modulate in vivo cytoxicity against tumor targets. Nat. Med. **6:** 443–446

116 Izumi Y., Xu L., di Tomaso E., Fukumura D. and Jain R. K. (2002) Tumour biology: herceptin acts as an anti-angiogenic cocktail. Nature **416:** 279–280

117 Allgayer H., Babic R., Gruetzner K. U., Tarabichi A., Schildberg F. W. and Heiss M. M. (2000) c-erbB-2 is of independent prognostic relevance in gastric cancer and is associated with the expression of tumor-associated protease systems. J. Clin. Oncol. **18:** 2201–2209

118 Nicolini G., Miloso M., Moroni M. C., Beguinot L. and Scotto L. (1996) Post-transcriptional control regulates transforming growth factor alpha in the human carcinoma KB cell line. J. Biol. Chem. **271:** 30290–30296

119 Sato Y., Roman M., Tighe H., Lee D., Corr M., Nguyen M. D. et al. (1996) Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. Science **19:** 352–354

120 Tsavaris N., Kosmas C., Vadiaka M., Kanelopoulos P. and Boulamatsis D. (2002) Immune changes in patients with advanced breast cancer undergoing chemotherapy with taxanes. Br. J. Cancer **87:** 21–27

121 Sfondrini L., Besusso D., Rumio C., Rodolfo M., Ménard S. and Balsari A. (2002) Prevention of spontaneous mammary adenocarcinoma in HER2/neu transgenic mice by foreign DNA. FASEB J. **16:** 1749–1754

122 Rilke F., Colnaghi M. I., Cascinelli N., Andreola S., Baldini M. T., Bufalino R. et al. (1991) Prognostic significance of HER2/*neu* expression in breast cancer and its relationship to other prognostic factors. Int. J. Cancer **49:** 44–49

123 Pupa S. M., Bufalino R., Invernizzi A. M., Andreola S., Rilke F., Lombardi L. et al. (1996) Macrophage infiltrate and prognosis in c-erbB-2-overexpressing breast carcinomas. J. Clin. Oncol. **14:** 85–94

124 Ménard S., Pupa S. M., Casalini P., Rilke F. and Colnaghi M. I. (1996) Relationship of histological grade, c-erbB-2 expression and inflammatory infiltrate to prognosis in carcinoma of the breast – in reply. J. Clin. Oncol. **14:** 2407

125 Pupa S. M., Ménard S., Andreola S. and Colnaghi M. I. (1993) Antibody response against the c-erbB-2 oncoprotein in breast carcinoma patients. Cancer Res. **53:** 5864–5866

126 Disis M. L., Calenoff E., McLaughlin G., Murphy A. E., Chen W., Groner B. et al. (1994) Existent T-cell and antibody immunity to HER2/neu protein in patients with breast cancer. Cancer Res. **54:** 16–20

127 Disis M. L., Pupa S. M., Gralow J. R., Dittadi R., Ménard S. and Cheever M. A. (1997) High titer HER2/neu protein specific antibody immunity can be detected in patients with early stage breast cancer. J. Clin. Oncol. **15:** 3363–3367

128 Disis M. L. and Cheever M. A. (1997) HER2/neu protein: a target for antigen-specific immunotherapy of human cancer. Adv. Cancer Res. **71:** 343–371

129 Foy T. M., Fanger G. R., Hand S., Gerard C., Bruck C. and Cheever M. A. (2002) Designing HER2 vaccines. Semin. Oncol. **29:** 53–61

130 Kiessling R., Weil W. Z., Herrmann F., Lindencrona J. A., Choudhury A., Kono K. et al. (2002) Cellular immunity to the Her-2/neu protooncogene. Adv. Cancer Res. **85:** 101–144

131 Rovero S., Amici A., Carlo E. D., Bei R., Nanni P., Quaglino E. et al. (2000) DNA vaccination against rat her-2/Neu p185 more effectively inhibits carcinogenesis than transplantable carcinomas in transgenic BALB/c mice. J. Immunol. **165:** 5133–5142

132 Di Carlo E., Rovero S., Boggio K., Quaglino E., Amici A., Smorlesi A. et al. (2001) Inhibition of mammary carcinogenesis by systemic interleukin 12 or p185neu DNA vaccination in Her-2/neu transgenic BALB/c mice. Clin. Cancer Res. **7:** 830s–837s

133 Nanni P., Nicoletti G., De Giovanni C., Landuzzi L., Di Carlo E., Cavallo F. et al. (2001) Combined allogeneic tumor cell vaccination and systemic interleukin 12 prevents mammary carcinogenesis in HER2/neu transgenic mice. J. Exp. Med. **194:** 1–12

134 Quaglino E., Iezzi M., Mastini C., Amici A., Pericle F., Di Carlo E. et al. (2004) Electroporated DNA vaccine clears away multifocal mammary carcinomas in HER2/neu transgenic mice. Cancer Res. **64:** 2858–2864

135 Lollini P. L. and Forni G. (2002) Antitumor vaccines: is it possible to prevent a tumor? Cancer Immunol. Immunother. **51:** 409–416

136 Nanni P., Forni G. and Lollini P. L. (1999) Cytokine gene therapy: hopes and pitfalls. Ann. Oncol. **10:** 261–266

137 Cavallo F., Di Carlo E., Butera M., Verrua R., Colombo M. P., Musiani P. et al. (1999) Immune events associated with the cure of established tumors and spontaneous metastases by local and systemic interleukin 12. Cancer Res. **59:** 414–421

138 Bocchia M., Bronte V., Colombo M. P., De Vincentiis A., Di Nicola M., Forni G. et al. (2000) Antitumor vaccination: where we stand. Haematologica **85:** 1172–1206

139 Finn O. J. and Forni G. (2002) Prophylactic cancer vaccines. Curr. Opin. Immunol. **14:** 172–177

140 Jager E., Jager D. and Knuth A. (2002) Clinical cancer vaccine trials. Curr. Opin. Immunol. **14:** 178–182

141 Knutson K. L., Schiffman K. and Disis M. L. (2001) Immunization with a HER2/neu helper peptide vaccine generates HER2/neu CD8 T-cell immunity in cancer patients. J. Clin. Invest. **107:** 477–484

142 Disis M. L., Gooley T. A., Rinn K., Davis D., Piepkorn M., Cheever M. A. et al. (2002) Generation of T-cell immunity to the HER2/neu protein after active immunization with HER2/neu peptide-based vaccines. J. Clin. Oncol. **20:** 2624–2632

143 De Vecchi R., Pupa S. M., Ménard S. and Lollini P.-L. (1999) Correspondence re: Y. Chen et al., DNA vaccines encoding full-length or truncated neu induce protective immunity against neu-expressing mammary tumors. Cancer Res. 5**8:** 1965–1971, 1998. Cancer Res. **59:** 4471–4472

144 Cefai D., Morrison B. W., Sckell A., Favre L., Balli M., Leunig M. et al. (1999) Targeting HER2/neu for active-specific immunotherapy in a mouse model of spontaneous breast cancer. Int. J. Cancer **83:** 393–400

145 Esserman L. J., Lopez T., Montes R., Bald L. N., Fendly B. M. and Campbell M. J. (1999) Vaccination with the extracellular domain of p185neu prevents mammary tumor development in neu transgenic mice. Cancer Immunol. Immunother. **47:** 337–342

146 Dakappagari N. K., Douglas D. B., Triozzi P. L., Stevens V. C. and Kaumaya P. T. (2000) Prevention of mammary tumors with a chimeric HER2 B-cell epitope peptide vaccine. Cancer Res. **60:** 3782–3789

147 Pupa S. M., Invernizzi A. M., Forti S., Nanni P., Lollini P. L., Meazza R. et al. (2001) Prevention of neu-expressing breast tumor development in mice transgenic for rat proto-neu by DNA vaccination. Gene Ther. **8:** 75–79

148 Press M. F., Cordon-Cardo C. and Slamon D. J. (1990) Expression of the HER2/neu proto-oncogene in normal human adult and fetal tissues. Oncogene **5:** 593–962
149 Ohashi P. S. and DeFranco A. L. (2002) Making and breaking tolerance. Curr. Opin. Immunol. **14:** 744–759
150 Prenzel N., Fischer O. M., Streit S., Hart S. and Ullrich A. (2001) The epidermal growth factor receptor family as a central element for cellular signal transduction and diversification. Endocr. Relat. Cancer **8:** 11–31
151 Sirotnak F. M., Zakowski M. F., Miller V. A., Scher H. I. and Kris M. G. (2000) Efficacy of cytotoxic agents against human tumor xenografts is markedly enhanced by coadministration of ZD1839 (Iressa), an inhibitor of EGFR tyrosine kinase. Clin. Cancer Res. **6:** 4885–4892
152 McCormick S. R., Lillemoe T. J., Beneke J., Schrauth J. and Reinartz J. (2002) HER2 assessment by immunohistochemical analysis and fluorescence in situ hybridization: comparison of HercepTest and PathVysion commercial assays. Am. J. Clin. Pathol. **117:** 935–943
153 Normanno N., Campiglio M., De Luca A., Somenzi G., Maiello M., Ciardiello F. et al. (2002) Cooperative inhibitory effect of ZD1839 ('iressa') in combination with trastuzumab ('herceptin') on human breast cancer cell growth. Ann. Oncol. **13:** 65–72
154 Moasser M. M., Basso A., Averbuch S. D. and Rosen N. (2001) The tyrosine kinase inhibitor zd1839 ('iressa') inhibits her2-driven signaling and suppresses the growth of her2-overexpressing tumor cells. Cancer Res. **61:** 7184–7188
155 Campiglio M., Locatelli A., Olgiati C., Normanno N., Somenzi G., Viganò L. et al. (2004) Inhibition of proliferation and induction of apoptosis in breast cancer cells by the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor ZD1839 ('Iressa') is independent of EGFR expression level. J. Cell Physiol. **198:** 259–268
156 Witters L., Engle L. and Lipton A. (2002) Restoration of estrogen responsiveness by blocking the HER2/neu pathway. Oncol. Rep. **9:** 1163–1166