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Role of ErbB2 and ErbB4 in Cancer Growth, Prognosis and as Targets for Immunotherapy

by

Maija Hollmén

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From the department of Medical Biochemistry and Genetics, and MediCity Research Laboratory, University of Turku, and Turku Graduate School of Biomedical Sciences, Turku, Finland

Supervised by

Professor Klaus Elenius, MD, PhD
Department of Medical Biochemistry and Genetics, and
MediCity Research Laboratory
University of Turku
Turku, Finland

Reviewed by

Docent Sirpa Leppä, MD, PhD
Department of Oncology
Helsinki University Central Hospital
Helsinki, Finland

and

Docent Cecilia Sahlgren, PhD
Department of Biology
Åbo Akademy University
Turku Centre for Biotechnology
Turku, Finland

Opponent

Professor Nancy E. Hynes, PhD
Friedrich Miescher Institute
Basel, Switzerland

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No amount of experimentation can ever prove me right;
a single experiment can prove me wrong
-Albert Einstein

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ABSTRACT

ErbB receptors (EGFR, ErbB2, ErbB3 and ErbB4) are growth factor receptors that regulate signals of cell differentiation, proliferation, migration and survival. Inappropriate activation of these receptors is associated with the development and severity of many cancers and has prognostic and predictive value in cancer therapy. Drugs, such as therapeutic antibodies, targeted against EGFR and ErbB2, are currently used in therapy of breast, colorectal and head and neck cancers.

The role of ErbB4 in tumorigenesis has remained relatively poorly understood. Alternative splicing produces four different isoforms of one ErbB4 gene. These isoforms (JM-a, JM-b, CYT-1 and CYT-2) are functionally dissimilar and proposed to have different roles in carcinogenesis. The juxtamembrane form JM-a undergoes regulated intramembrane proteolysis producing a soluble receptor ectodomain and an intracellular domain that translocates into the nucleus and regulates transcription. Nuclear signaling via JM-a isoform stimulates cancer cell proliferation.

This study aimed to develop antibodies targeting the proposed oncogenic ErbB4 JM-a isoform that show potential in inhibiting ErbB4 dependent tumorigenesis. Also, the clinical relevance of ErbB4 shedding in cancer was studied. The currently used monoclonal antibody trastuzumab, targeting ErbB2, has shown efficacy in breast cancer therapy. In this study novel tissues with ErbB2 amplification and trastuzumab sensitivity were analyzed.

The results of this study indicated that a subpopulation of breast cancer patients demonstrate increased shedding and cleavage of ErbB4. A JM-a isoform-specific antibody that inhibited ErbB4 shedding and consequent activation of ErbB4 had anti-tumor activity both *in vitro* and *in vivo*. Thus, ErbB4 shedding associates with tumor growth and specific targeting of the cleavable JM-a isoform could be considered as a strategy for developing novel ErbB-based cancer drugs. In addition, it was demonstrated that ErbB2 amplification is common in intestinal type gastric cancers with poor clinical outcome. Trastuzumab inhibited growth of gastric and breast cancer cells with equal efficacy. Thus, ErbB2 may be a useful target in gastric cancer.

Key words: ErbB2, ErbB4, JM-a isoform, shedding, cancer, therapeutic antibodies

Maija Hollmén
ErbB2- ja ErbB4-reseptorien rooli syövän kasvussa, ennusteessa ja immunoterapian kohdeproteiineina
Lääketieteellinen biokemia ja genetiikka, MediCity-tutkimuslaboratorio, Turun yliopisto, Turun biolääketieteellinen tutkijakoulu (TuBS), Turku
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TIIVISTELMÄ

ErbB-reseptorit (EGFR, ErbB2, ErbB3 ja ErbB4) ovat kasvutekijäreseptoreita, jotka säätelevät solun kasvua, jakautumista, erilaistumista ja selviytymistä edistäviä signaaleja. ErbB-reseptorien yliaktiivista toimintaa esiintyy monissa ihmisen pahanlaatuisissa kasvaimissa, ja tällä on todettu olevan sekä ennusteellista että hoidollista merkitystä. Tällä hetkellä EGFR ja ErbB2-reseptorien toimintaa estäviä lääkkeitä, kuten monoklonaalisia vasta-aineita, on käytössä rintasyövän, paksusuolensyövän sekä pään ja kaulan alueen syöpien hoidossa.

ErbB4-reseptorin rooli syövässä on edelleen epäselvä. ErbB4-geenistä tehdään vaihtoehtoisen silmukoinnin kautta neljää eri alamuotoa. Alamuodot (JM-a, JM-b, CYT-1 ja CYT-2) eroavat sekä rakenteeltaan että toiminnaltaan toisistaan ja niillä on osoitettu olevan erilaiset vaikutukset syövän kehittymiseen. Muista alamuodoista poiketen jukstamembraaninen JM-a–alamuoto prosessoidaan solukalvolla proteolyyttisen aktiivisuuden seurauksena liukoiseksi reseptorin solunulkoiseksi osaksi ja solunsisäiseksi osaksi. Reseptorin solunsisäinen osa kulkeutuu tumaan, missä se säätelee geenien luentaa. Tämän alamuodon on todettu edistävän syöpäsolujen jakautumista.

Tutkimuksen tarkoituksena oli kehittää ja karakterisoida vasta-aine, joka spesifisesti tunnistaa JM-a alamuodon ErbB4-reseptorista sekä estää reseptorin katkeamista kudoksessa vähentäen näin tumaan menevän solunsisäisen reseptoriosan määrää, ja selvittää mikä vaikutus kyseisellä aineella on syövän kasvuun ja kehitykseen. Koska ErbB2 vasta-aine, trastutsumabi, on osoittautunut tehokkaaksi rintasyövän hoidossa. Tutkimuksessa selvitettiin myös muita mahdollisia ErbB2-monistuman sisältämiä syöpäkudoksia ja niiden herkkyyttä trastutsumabille.

Tutkimuksen tulokset osoittivat, että ErbB4 JM-a-alamuoto on lupaava kohdemolekyyli ErbB-reseptoreiden signaloinnin terapeuttisessa estämisessä. JM-a-alamutospesifisellä vasta-aineella pystyttiin tehokkaasti estämään syöpäsolujen jakautumista ja kasvainten muodostusta. Lisäksi osoitimme, että ErbB2-monistuma on yleinen huonon ennusteen intestinaalityyppisessä mahasyövässä ja mahasyöpäsolut ovat yhtä herkkiä trastutsumabille kuin ErbB2-positiiviset rintasyöpäsolut. Näin ollen ErbB2-reseptorin toimintaa estävää lääkitystä olisi myös mahdollista käyttää mahasyövässä.

Avainsanat: ErbB2, ErbB4, JM-a-alamuoto, katkeaminen, syöpä, terapeuttiset vasta-aineet

TABLE OF CONTENTS

ABBREVIATIONS

ADAM	A disintegrin and a metalloprotease
ADCC	Antibody-dependent cell cytotoxity
ATP	Adenosine trisphosphate
AR	Amphiregulin
BAD	Bcl-2 associated death domain
BTC	Betacellulin
CDR	Complementary determining region
CHO	Chinese hamster ovary
CISH	Chromogenic in situ hybridization
COX-2	Cyclooxygenase 2
CRC	Colorectal carcinoma
CYT	Cytoplasmic
DAG	Diacylglycerol
ECD	Extracellular domain
ECM	Extracellular matrix
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EPG	Epigen
EPR	Epiregulin
ER	Estrogen receptor
ERK	Extracellular-regulated kinase 1, 2
FAK	Focal adhesion kinase
FDA	Food and drug administration, USA
FISH	Fluorescence in situ hybridization
FOXO1	Forkhead box O transcription factor
GSK3β	Glycogen synthase kinase-3β
HNSCC	Head and neck squamous cell carcinoma
HB-EGF	Heparin-binding EGF-like growth factor
HER	Human epidermal growth factor receptor
Ig	Immunoglobulin
IHC	Immunohistochemistry
ICD	Intracellular domain
IP_3	Inositol triphosphate
JM	Juxtamembrane
kDa	Kilodalton
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MVB	Multivesicular body
NLS	Nuclear localization signal
NRG	Neuregulin
NSCLC	Non-small cell lung cancer
PDK1	3-phosphoinositide-dependent kinase 1
PIP_2	Phosphatidylinositol (4,5)-biphosphate
PIP_3	Phosphatidylinositol-3,4,5-triphosphate
PI3-K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PLC-γ	Phospholipase C-gamma
PMA	Phorbol 13-myristate 12-acetate
PTEN	Phosphatase and tensin homologue
PTB	Phosphotyrosine-binding
PTK	Protein tyrosine kinase
RIP	Regulated intramembrane proteolysis
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription-polymerase chain reaction
SCC	Squamous cell carcinoma
SH2	Src homology 2
siRNA	Small interfering RNA
STAT	Signal transducer and activator of transcription
TACE	Tumor necrosis factor-alpha converting enzyme
TGF-α	Transforming growth factor
mTOR	Mammalian target of rapamycin
TPA	12-*O*-tetradecanoylphorbol-13-acetate
Wwox	WW domain-containing oxidoreductase
YAP	Yes-associated protein

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which will be referred to by their Roman numerals (I-III).

I M Hollmén, J Määttä, L Bald, MX Sliwkowski and K Elenius, Suppression of breast cancer cell growth by a monoclonal antibody targeting cleavable ErbB4 isoforms, Oncogene 2009, 28:1309-19.

II M Hollmén, I Reinvall, T Vahlberg, A Smeets, T van Dorpe, H Joensuu, H Wildiers, P Schöffski and K Elenius, ErbB4 ectodomain as a biomarker and potential therapeutic target in breast cancer, Manuscript.

III M Tanner, M Hollmén, TT Junttila, S Tommola, Y Soini, H Helin, J Salo, H Joensuu, E Sihvo, K Elenius and J Isola, Amplification of HER-2 in gastric carcinoma: association with Topoisomerase IIalpha gene amplification, intestinal type, poor prognosis, and sensitivity to trastuzumab, Annals of Oncology 2005, 16:273-278.

1 INTRODUCTION

The ErbB/HER subfamily of receptor tyrosine kinases includes four members: EGFR (also known as ErbB1 or HER1), ErbB2 (c-Neu, HER2), ErbB3 (HER3), and ErbB4 (HER4). ErbB receptors are selectively activated by a number of EGF-like growth factors leading to cellular responses, such as proliferation, differentiation, migration, or survival. Overactive signaling of ErbB receptors is commonly implicated in malignancies of epithelial and neural origin, and ErbB expression is typically associated with poor clinical outcome. Specific abnormalities in gene expression and mutational status provide prognostic and predictive value, and these alterations form the basis for therapeutics aiming at inhibiting ErbB activity in cancer. The successful clinical targeting of EGFR and ErbB2 by monoclonal antibodies in several cancer types highlights the relevance of targeting other diseased tissues that possibly harbor abnormal ErbB receptor activities.

Although less is understood about the abnormal activities of ErbB4, it is frequently present in various cancer tissues, and cancer-associated mutations in the ErbB4 gene have recently been described. However, the biology of ErbB4 is complicated when compared to EGFR or ErbB2 as ErbB4 is expressed as four alternatively spliced and functionally distinct isoforms. Current evidence of the oncogenic potential of ErbB4 propose both growth promoting as well as differentiation activities in various cancer models and human cancer samples. Recently there has been more emphasis on resolving the individual roles of the ErbB4 isoforms in tumorigenesis. In conclusion the ErbB4 isoforms have been proposed to promote or suppress tumorigenesis depending on their isoform specific activities. One of the ErbB4 isoforms (JM-a) undergoes regulated intramembrane proteolysis that results in transcriptional regulation by the nuclear receptor fragment. Nuclear ErbB4 is associated with growth promoting functions and relatively unfavorable prognosis in breast cancer.

The aim of this study was to develop new tools and discover novel target tissues to inhibit ErbB-dependent tumorigenesis and to find out the significance of ErbB4 expression and cleavage in cancer biology and disease prognosis. The successful clinical use of monoclonal antibodies in cancer therapy and their target specificity provided the basis for conducting this study.

2 REVIEW OF THE LITERATURE

2.1 Cancer

2.1.1 Malignant transformation

Normal cell behavior is strictly controlled to sustain a homeostasis between life and death. During malignant transformation a cell gains several features that enables the cell to proliferate uncontrolled and independently of extrinsic factors. These features can be a result of carcinogens, such as tobacco smoke, radiation, chemicals, infectious agents or naturally occurring mutations that cause genomic instability and abnormal cell metabolism. The epigenetic changes in DNA methylation (Jones and Laird 1999) and dysregulated microRNAs (Volinia et al 2006) importantly add to the complexity of the initiation and progression of malignant transformation.

It is thought that neoplastic transformation is a multi-step process involving changes in growth factor sensitivity, differentiation state, apoptosis, angiogenesis and metastasis (Hanahan and Weinberg 2000). The prevalence of dominant oncogenes in various cancers clearly defines the importance of acquired growth signal autonomy of a transformed cell (Kranenburg 2005, Prochownik 2008, Slamon et al 1987). The strategies by which a cell reduces its dependence on external stimulation include self-sufficient production of growth factors. Receptors, which mediate the signals of growth factors, often harbor a kinase enzyme that can be activated by overexpression, structural changes or mutations increasing the level of signal autonomy. Another means to sustain malignant growth is to co-operate with neighboring cells so that they maintain a favorable microenvironment. Inflammatory cells attracted to sites of neoplasia may promote rather than eliminate malignant growth (Hagemann et al 2009).

Various antiproliferative signals act to balance the growth promoting effects of oncogenes. In normal conditions tumor suppressor gene products monitor their environment and decide whether to instruct the cells to proliferate, maintain quiescence or enter into a post-mitotic state. For a cancer cell to maintain its proliferative phenotype it needs to bypass any signals that drive it towards terminal differentiation. The acquired capability to evade apoptosis is also important in maintaining the survival of a transformed cell. The apoptotic machinery can be divided into sensors and effectors. The sensors mediate signals from the external and internal environment for the effectors to execute selective destruction of the genome and cellular organelles. The p53 tumor suppressor gene is a key component of the proapoptotic regulation of DNA damage and mutations in this gene occur frequently in human cancers (Vazquez et al 2008).

Regardless of significantly altered proliferation rates observed in malignant transformation a cell can only progress through a certain number of doublings until it stops growing. In every cell division a short repeat of telomere DNA is removed from the chromosomal ends that results from the inability of DNA polymerases to completely replicate the 3' ends of chromosomal DNA. The progressive erosion of telomeres eventually leads to end-to-end fusions of chromosomes, cell crisis and cell death. The immortalized cell, however, has the ability to maintain its telomeres in a length that permits unlimited replication.

The replicative tumor cells, like normal tissues, are dependent on oxygen and nutrients supplied by blood vessels from close vicinity. Tumor angiogenesis is a prerequisite for the formation of macroscopic tumors over 1 mm in diameter and can be induced and sustained via an angiogenic switch from vascular quiescence by a shift in the balance between angiogenic activators and inhibitors present in the tumor microenvironment.

2.1.2 Invasion and metastasis

Most types of cancers can acquire the capability to invade into adjacent tissue and travel to distant sites of the human body causing 90 % of cancer deaths in human (Sporn 1996). Invasion and metastasis are extremely complex processes. Changes in several classes of proteins that regulate cell morphology, cell-cell or cell-extracellular matrix (ECM) adhesion interactions, and matrix remodeling have been detected to contribute to tumor cell spreading. The alteration in normal ECM composition of collagens, fibronectins, laminins or proteoglycans, increases ECM stiffness, which in turn increases cell contractility by clustering and activating integrins. The altered integrin signaling and subtype expression increases the expression of certain kinases and growth factors resulting in the disruption of normal tissue architecture and the promotion of invasion (Paszek et al 2005).

The detached tumor cell forms protrusive processes known as invadopodia that facilitate initial digestion and invasion of the ECM. The structure and function of invadopodia are somewhat similar to the structure of filopodia observed during two-dimensional migration, and require highly localized and coordinated actin polymerization. The structure of invadopodia, however, can spatially focus proteolytic secretion, thereby facilitating the remodeling of the ECM (Yamaguchi et al 2005). The removal of the ECM liberates the invasive cell to enter the vasculature and adhere to the endothelium of a target tissue. In order to form colonies at different sites the adherent cell undergoes diapedesis, a process of cytoskeletal remodeling that enables the cell to penetrate the cell-cell junctions in the endothelium (Miles et al 2008).

2.1.3 Targeted cancer therapeutics

For decades cancer treatment has focused on eliminating tumor cells by surgery, chemotherapy, hormonal therapy and radiation. Although these treatments have developed over the years they still have their limitations and the search for more efficient and non-toxic treatments is prevailing. The main problems in currently used chemotherapeutic agents are their frequently occurring severe side effects and rapid development of drug resistance. These are caused by the untargeted cytotoxic effects towards all proliferating tissues as well as disruption of DNA synthesis and repair, respectively. Advances in molecular biology have led to the identification of specific abnormalities responsible for tumor progression that serve as targets for improved cancer therapeutics. The key discoveries include the recognition of cancer specific antigens (e.g. tyrosine kinases and other enzymes), understanding disease pathways and development of means for specifically targeting diseased cells.

The evolutionary steps that a cell takes to become malignant are highly variable. The acquisition of malignant features can differ in order, amount and stage of progression even in histologically identical tumors. All features described in previous chapters of tumorigenesis offer various ways to target the multi-step process of transformation. In some cases a single genetic event can lead to the progression of tumorigenesis. For example, one such event is the fusion of two genes from different chromosomes by reciprocal translocation producing abnormal tyrosine kinase activity of the resulting fusion protein BCR-ABL. This hallmark was identified as the cause of chronic myeloid leukemia and led to the development of imatinib (Glivec), a tyrosine kinase inhibitor that was successful in the treatment of patients with this chromosomal abnormality (Druker et al 2001). Another well characterized oncogenic alteration is the amplification of the ErbB2 gene in breast cancer causing overactive growth-promoting signaling of the ErbB2 tyrosine kinase enzyme (Slamon et al 1987). Strategies to inhibit ErbB2-dependent growth resulted in the development of a monoclonal antibody trastuzumab (Herceptin) that specifically blocks the functions of this receptor in tissues harboring amplification (Hudis 2007). Trastuzumab increases the overall survival in ErbB2-positive breast cancer (Slamon et al 2001). Imatinib and trastuzumab were among the first new-age anticancer drugs to get approval from FDA. Oncogene-targeted therapeutic strategies have also been shown to sensitize tumor cells to and act synergistically with the conventional anticancer therapies (O'Rourke et al 1998), prevent multiple malignant activities including angiogenesis (Petit et al 1997) and reverse oncogene-associated immune resistance of the tumor by antibody-dependent cell cytotoxity (ADCC) (Clynes et al 2000).

2.1.3.1 Biomarkers in cancer

As much as the molecular targets in cancer offer drug development opportunities they also provide crucial information for disease prevention, prognosis, treatment efficacy and resistance. Molecules expressed by malignant cells serve as indicators of the alterations occurring in disease progression and can be monitored from various sites of the human body. Tissue biopsies from malignant lesions are often exploited for their expression of specific biomarkers for a given cancer. ER-positivity in breast cancer is a marker of favorable disease outcome and offers a predictive biomarker for hormonal therapies, whereas ErbB2 receptor-positivity associates with estrogen receptor negativity, shorter survival, metastasis, and predicts sensitivity to anti-ErbB2 therapies (Payne et al 2008). Tumor-associated mutations in the EGFR kinase domain are markers for lung cancer patients who benefit from the EGFR tyrosine kinase inhibitor therapy (Sharma et al 2007). The somatic mutations in KRAS, in contrast, associate with resistance to EGFR targeted agents in metastatic colorectal cancer. In fact, patients treated with chemotherapy and cetuximab (EGFR antibody) that have mutated KRAS actually show decreased progression-free survival when compared to patients receiving only chemotherapy (Tol et al 2009).

Tissue biopsies are always invasive why active tissue biomarker-based disease follow-up is difficult. Malignant lesions may also reside at locations that are not accessible. Circulating biomarkers offer additional information for monitoring cancer and include a wide category of tumor-associated antigens, secreted proteins that can be detected from serum, urea, saliva and other body fluids. These proteins often share high tumor specificity and sensitivity, and are commonly used in the management of various cancers. EGFR and ErbB2 extracellular domains have been analyzed as soluble biomarkers from patient serum for prognostic information and monitoring the efficacy of ErbB-based therapies. The tumor-induced release of many proteins at abnormal levels can lead to the activation of the immune system and the production of autoantibodies (Mintz et al 2003). Such antibodies can be used to detect patients with cancers prior to the clinical diagnosis. The anticipated benefit of these early biomarkers is to detect lesions in premalignant stage when the disease treatment is often curative. Other circulating biomarkers include cell-free DNA and cancer cells. Elevated levels of cell-free DNA may result from active cell destruction via necrosis and is an indicator of tumor progression and lymph node involvement (Umetani et al 2006). Circulating tumor cells detected in patients suffering from either localized or metastatic breast cancer have been associated with poor outcome (Cristofanilli et al 2004).

2.2 ErbB receptors

2.2.1 Structure

Tyrosine phosphorylation is a covalent modification used for intercellular communication in multicellular organisms. The enzymes catalyzing the transfer of the γ-phosphate of adenosine triphosphate (ATP) to tyrosine residues on protein substrates are called protein tyrosine kinases (PTKs) (Hubbard and Till 2000). There are over 90 genes in the human genome encoding PTKs and 58 of them code for transmembrane receptor tyrosine kinases (RTK) (Blume-Jensen and Hunter 2001). The RTKs form 20 subfamilies and comprise a large fraction of dominant oncogenes (Hunter 1997). ErbB RTK subfamily includes EGFR, also known as ErbB1 or HER1 (Carpenter et al 1975, Ullrich et al 1984), ErbB2 (neu, HER2) (Bargmann and Weinberg 1988, Stern et al 1986), ErbB3 (HER3) (Plowman et al 1990) and ErbB4 (HER4) (Plowman et al 1993). They are heavily glycosylated membrane spanning tyrosine kinase receptors of approximately 180 kDa in size. The receptor sequence can be divided into different domains according to structural and functional variations. All ErbB receptors have an extracellular domain for ligand binding properties, a single transmembrane domain (TM) and an intracellular domain containing a tyrosine kinase enzyme and a C-terminal domain for mediating signal transduction (Figure 1).

The extracellular domain (~620 residues) consists of four distinct domains of two different types in the order L1-CR1-L2-CR2 (Ward and Garrett 2001) also named domains I-II-III-IV (Lax et al 1988). The large (L) domains are members of the leucine rich repeat (LRR) family and adopt a right-handed β-helix. The other two domains are cysteine-rich (CR) domains formed from multiple small disulfide-bonded modules that take part in receptor dimerization and activation of the tyrosine kinase (Berezov et al 2002). The transmembrane domain (~23 residues) is an α-helix structure containing potential GXXXG dimerization motifs (Mendrola et al 2002). The TM domain has been shown to be an important interacting motif in ligand activated dimerization as a specific Val → Glu mutation in the Neu oncogene product within the TM domain is known to induce ErbB2 dimerization and activation (Bargmann and Weinberg 1988, Weiner et al 1989). The juxtamembrane domain (~40 residues) in the intracellular part of ErbB receptors is located right after the TM domain. This region appears to have a number of regulatory functions in the events of downregulation and ligand-dependent internalization (Kil and Carlin 2000), as well as basolateral sorting of EGFR (He et al 2002). In addition, the juxtamembane region contains a nuclear localization sequence (NLS) that mediates the nuclear localization of ErbB receptors (Hsu and Hung 2007). The kinase domain (~260 residues) adopts the bilobate-fold characteristic of all previously reported PTKs. It consists of the NH_2-terminal lobe (N-lobe) formed

mostly by β-strands and one α-helix (αC) and a larger C-terminal lobe (C-lobe) harboring important elements of the catalytic machinery. The N-lobe and C-lobe are separated by a cleft in which ATP can bind. The opening angles between these two lobes are important in bringing the phosphate-binding loop from the N-lobe and the catalytic and activation loop (A-loop) from the C-lobe in proximity (Stamos et al 2002). The carboxy-terminal domain (~232 residues) contains several tyrosine residues that provide specific docking sites for the intracellular signal transducers and adaptors upon phophorylation. The C-terminal end has been identified to bind actin and take part in the formation of ligand activated receptor clustering (den Hartigh et al 1992).

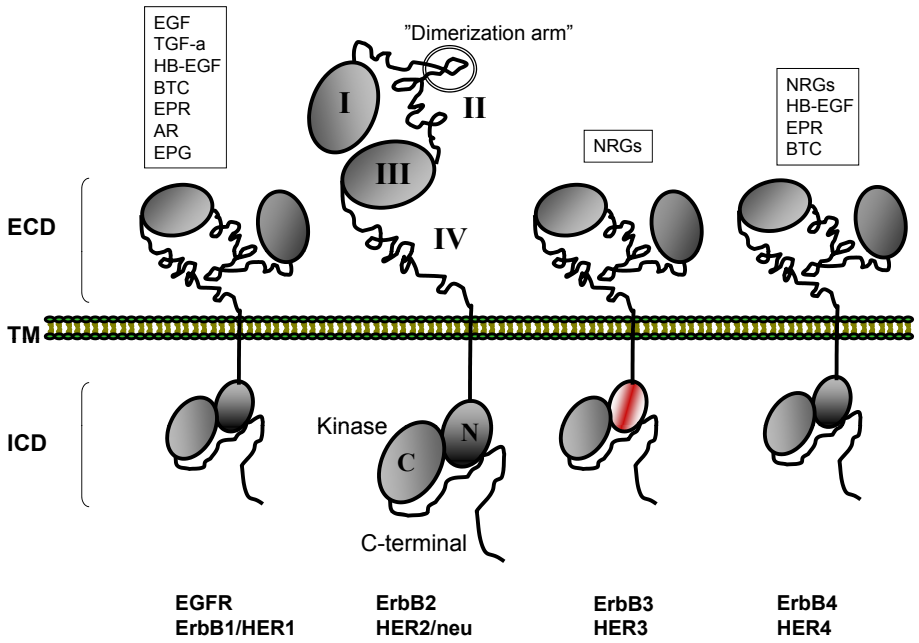

Figure 1. Structures and ligands of the ErbB family receptors.
ErbB receptors consist of an extracellular domain (ECD), a single transmembrane (TM) spanning alpha helix, and an intracellular domain (ICD). A closer up view of ErbB2 with the specific structures: the ECD is divided into four domains of different functions. Domains I and III take part in ligand binding and domains II and IV provide the specific interface for receptor dimerization. The ICD is divided into the juxtamembrane domain, the kinase domain and the C-terminal domain. The kinase is formed by two different structures named the N-lobe and C-lobe. The ErbB receptor specific ligands are listed above each receptor. Different from the other ErbBs, ErbB2 does not bind any known ligand and is found to adopt an extended conformation where the dimerization arm is exposed to interact with other family members. ErbB3 is kinase impaired due to structural changes in the N-lobe and can only signal as a heterodimer.

2.2.2 Ligands

There are several ligands identified to mediate their signals through ErbB receptors. The epidermal growth factor (EGF), transforming growth factor-α (TGF-α), amphiregulin (AR), heparin-binding EGF like growth factor (HB-EGF), betacellulin (BTC), epiregulin (EPR) and epigen (EPG) all bind and activate EGFR (Riese et al 1996a, Strachan et al 2001). BTC, EPR and HB-EGF have also been identified to act by binding ErbB4 (Elenius et al 1997b, Riese et al 1996b). The family of neuregulins (NRG-1, NRG-2, NRG-3, NRG-4) solely activates ErbB4 with the exception of NRG-1 and NRG-2 that also bind ErbB3 (Carraway et al 1997, Chang et al 1997, Harari et al 1999, Zhang et al 1997) (Figure 1). All ErbB ligands have a consensus sequence that is crucial for binding members of the ErbB family and consists of six spatially conserved cysteine residues known as the EGF motif (Harris et al 2003). The ligands exist as membrane-anchored precursor proteins from which they are released by metalloprotease activity for paracrine and autocrine signaling (Lee et al 2003). All these ligands interact with receptors in identical fashion but induce distinct biological responses. The different responses can partly be explained by the formation of receptor homo- and heterodimers affecting ligand-binding affinities. The signaling from endosomes is also different from that generated at the cell surface as TGF-α has been shown to rapidly dissociate within the acidic mileau of the endosomes (French et al 1995) and be able to activate signaling pathways solely at the cell surface. EGF interacts with its receptor in the endosomes maintaining active EGFR-Ras signaling throughout early endosomal sorting (Haugh and Meyer 2002). The ErbB ligands TGF-α, HB-EGF, AR and NRG-1 have also been demonstrated to induce cellular functions in a juxtacrine fashion in which membrane-anchored growth factors transmit signals to neighboring cells (Anklesaria et al 1990, Bao et al 2003, Iivanainen et al 2007). This type of signaling is tightly regulated and can form bidirectional signaling between the growth factor and its receptor (Bao et al 2003, Iivanainen et al 2007).

2.2.3 Mechanism of action

The ErbB extracellular domains exist in two different conformations depending on ligand occupancy of the receptor. The unligated receptor adopts a tethered autoinhibited conformation where a loop from domain II called the "dimerization arm" forms an interaction with the modules in domain IV (Bouyain et al 2005, Cho and Leahy 2002, Ogiso et al 2002) (Figure 2). This interaction stabilizes the receptor and holds the ligand binding sites in domain I and III apart but exposed. Ligand binding to one of the I/III domains destabilizes the tether, resulting in a dramatic reconfiguration of the ectodomain to form the exposed active conformation (Garrett et al 2002). In the active conformation a unit of domains I and II rotate around domain III so that the ligand becomes clamped between domains I and III for high affinity ligand

binding. The dimerization arm in domain II and domain IV get exposed and positioned to interact with another activated receptor-ligand complex to form dimers (Burgess et al 2003) (Figure 2).

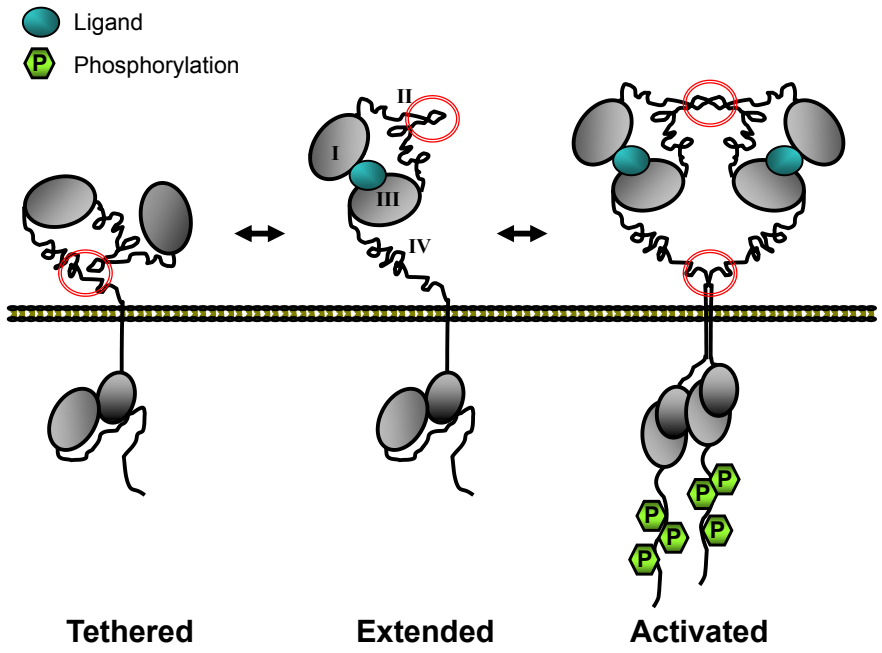

Figure 2. Activation of ErbB receptors
ErbB receptors apart from ErbB2 adopt an inactive tethered conformation when unoccupied by a ligand. The binding of a ligand to one of the ligand binding domains I or III breaks the dimerization arm contact between domains II and IV and rotates domains I and II so that the ligand gets into contact with both domains I and III for high affinity binding. The extended conformation exposes the dimerization arm and domain IV for contacting another extended ErbB receptor to form a dimer (important conformational parts are indicated with a circle). The dimerization event allows the intracellular kinase domains to allosterically activate each other by autophosphorylation resulting in the phosphorylation of tyrosine residues in the C-terminal region.

Mutations (Ferguson et al 2003) or deletions (Elleman et al 2001) that abolish the domain II/IV tether increase EGFR affinity for EGF or TGF-α by up to 30-fold. However, disruption of the tether causes no elevation in EGFR autophosphorylation in the absence of the ligand. Thus, these mutations are not sufficient to promote dimerization by simply exposing the dimerization arm in domain II (Mattoon et al 2004, Walker et al 2004). The ErbB2 ectodomain is constitutively in the active conformation due to sequence differences in domain IV affecting the formation of the tether interface between domains II and IV (Cho and Leahy 2002) (Figure 1). These contact regions are conserved in EGFR

and ErbB4, however, in ErbB2 three of the seven residues are different (Glu259 for Gln252, Pro572 for Gly563, and Phe574 for His565) (Cho and Leahy 2002). The opposing domains I and III in ErbB2 make direct contact with each other preventing any ligand binding to the extracellular domain of ErbB2 (Garrett et al 2003). These alterations liberate the dimerization arm and the complementary regions of domain IV to interact with other ligand-activated ErbB receptors (Berezov et al 2002). Although the ErbB2 receptor is apparently ready to form dimers, the constitutive homodimerization of ErbB2 is weak due to negative electrostatic forces of its dimerization arm (Garrett et al 2003). Thus, ErbB2 is the preferred heterodimerization partner for the other ErbB receptors in the activation of ErbB signaling (Graus-Porta et al 1997, Pinkas-Kramarski et al 1996). Interestingly, the structure of ErbB2 resembles the autoinhibited structure of the epidermal growth factor receptor in Drosphila melanogaster (dEGFR) (Alvarado et al 2009). In Drosophila the dEGFR is tightly regulated by growth factor ligands and instead of an intramolecular tether, the dEGFR is maintained inactive by a distinct set of autoinhibitory interdomain interactions also present in ErbB2 (Alvarado et al 2009). This argues against the suggestion that ErbB2 lacks autoinhibition (Garrett et al 2003).

The dimerization event typically activates a kinase domain by autophosphorylation of the activation loop. The ErbB receptors do not require such activation as a mutation in the tyrosine residue Y845 within the activation loop of EGFR does not change its kinase activity (Gotoh et al 1992). In fact, EGFR and ErbB4 are activated by asymmetric intermolecular allosteric interactions releasing the autoinhibited conformation for active catalysis (Qiu et al 2008, Zhang et al 2006). In the autoinhibited conformation the orientation of αC-helix from the N-lobe moves a conserved glutamate (Glu738) out of the active site and breaks the catalytically important salt bridge between the side chain of Glu738 and a conserved Lysine (721) in EGFR. The displacement of αC-helix is sterically coupled to the closed conformation of the activation loop preventing any substrate peptide binding to the active site (Bose and Zhang 2009). Regions from the intracellular juxtamemrane domain (residues 645-657) and a part of the C-terminal end (residues 971-979) of EGFR maintain the autoinhibited conformation by directly regulating the formation of the allosteric asymmetric kinase domain dimer (Thiel and Carpenter 2007, Wood et al 2004). However, a recent report by Red Brewer et al shows evidence that the juxtamembrane domain, instead of inhibiting the kinase, actually activates it. The juxtamembrane domain makes extensive contacts with the C-lobe of a donor monomer and stabilizes the dimer (Red Brewer et al 2009). In the activated dimer, the C-lobe of monomer A contacts the N-lobe of an adjacent monomer B and promotes conformational changes that activates the kinase of monomer B (Zhang et al 2006). The asymmetric dimer interfaces are conserved among EGFR, ErbB2 and ErbB4. ErbB3 also has a high sequence homology in the C-lobe regions of the dimer interface but not in the N-lobe face. The

conserved C-lobe allows ErbB3 to activate other members of the ErbB receptors by heterodimerization (Yarden and Sliwkowski 2001). ErbB3 on its own, however, is a catalytically inactive kinase due to several mutations in key regions of the active site preventing any signaling from ErbB3 homodimers (Guy et al 1994).

2.2.4 Signaling

Upon ErbB receptor activation the C-terminal tyrosine residues get phosphorylated and recruit several signal transducer and adaptor molecules to directly associate with the receptor by their Src homology 2 (SH2) or phosphotyrosine-binding (PTB) domains (Schlessinger 2000). The diversity in the C-terminal sequences of ErbB receptors allows different proteins to selectively associate with each receptor. Signaling proteins that are known to directly recognize phosphorylated tyrosines on EGFR include Grb-2, Shc, Dok-R, phospholipase Cγ (PLC-γ), PTB-1B, SHP-1, Src, and Abl (Jorissen et al 2003). The physical association of EGFR with adaptor proteins increases substrate phosphorylation and aids the assembly of spatially organized multicomponent signaling (Koch et al 1991). Some adaptor proteins interact with each other and with a subset of other cellular proteins apart from ErbB-dependent pathways due to their modular construction of several SH2 and PTB domains. For example, Grb2 has been shown to interact with focal adhesion kinase (FAK) to integrate the integrin and growth factor signals in promoting cytoskeletal reorganization (Sieg et al 2000). This greatly enhances the multiplicity of signals emanating from activated ErbB homo- and heterodimers. The stimulation of ErbB receptors results in the activation of multiple signaling pathways often functionally interlinked to each other (Figure 3).

2.2.4.1 MAPK

The Ras/mitogen-activated protein kinase (MAPK) pathway mediating proliferation, differentiation, migration and apoptosis is initiated by the relocation of Grb2 from the cytosol to the cell membrane to bind phosphorylated ErbB receptors. Grb2 is constitutively bound to the Ras exchange factor Sos and the movement of this complex to the membrane facilitates the interaction of membrane-associated Ras with Sos and results in the exchange of GDP to GTP in the activated Ras (Lowenstein et al 1992). The active Ras phosphorylates several other kinases such as Raf-1 (Hallberg et al 1994) leading to the activation of MEK (=MAPKK) and nuclear translocation of Erk-1 and Erk-2 (=MAPK), which in turn activate several transcription factors (Johnson and Vaillancourt 1994). Overactive MAPK pathway signaling is frequently observed in tumorigenesis and can be activated by mutated EGFR, Ras or Raf (Roberts and Der 2007).

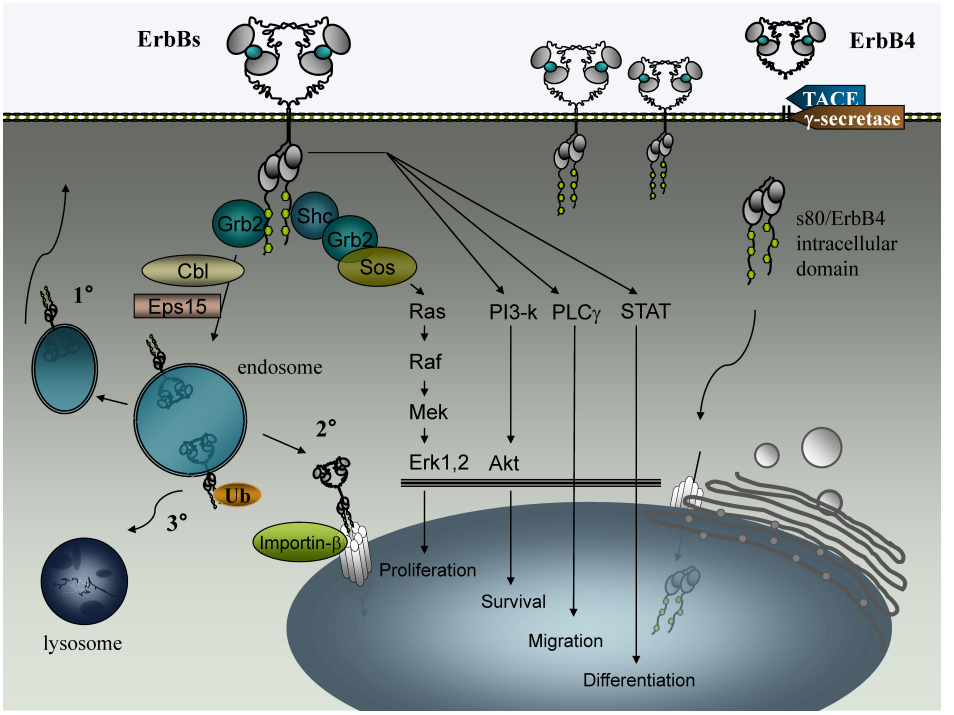

Figure 3. ErbB receptor signaling and endocytotic trafficking.
Upon phosphorylation the C-terminal residues of ErbB receptors recruit several signaling molecules either directly (PI3-K, PLCγ, STAT) or via adaptor molecules (Shc, Grb2) to mediate cellular functions in proliferation, survival, migration and differentation. ErbB receptors can also signal directly to the nucleus as full-length receptors (EGFR, ErbB2, ErbB3) via the endocytic machinery and importin-β- mediated nuclear transport or by regulated intramembrane proteolysis (RIP) (ErbB4). ErbB4 is the only ErbB family member to undergo RIP, a two-step proteolytic cleavage by TACE and γ-secretase activity resulting in a soluble 80 kDa intracellular domain fragment that can translocate into the nucleus and regulate transcription. The ligand activated ErbB dimers are endocytosed and trafficked through the endocytotic machinery and end up recycled (1°), transported to the nucleus (2°) or degraded (3°). The fate of the receptor depends on the molecules it recruits. In the case of EGFR, the activated receptor recruits Cbl that leads to its ubiquitination (Ub) and association with EPS15 for lysosomal targeting.

2.2.4.2 Src

The cytosolic Src family of kinases has also been implicated in the signal transduction of EGFR. It is not clear whether Src is signaling downstream of EGFR or contributing to its activation. The relationship of these molecules is thought to be bidirectional as Src kinase overexpression strongly enhances EGF-mediated transformation (Maa et al 1995) whereas specific tyrosine kinase inhibitors for EGFR reduce elevated kinase activity of Src to a basal level (Mao

et al 1997). Src kinase activity is required for the ErbB family activation of STAT (Olayioye et al 1999) and PI3-K (Stover et al 1995) pathways.

2.2.4.3 STATs

The STATs were originally identified as signal transducers of several cytokines including growth hormone, prolactin and erythropoietin. The activation of STATs by cytokines occurs through ligand-induced recruitment, and activation of the intracellular JAK kinases. There are seven STAT proteins of which STAT1, 3, 5a and 5b can also be activated by EGFR/Src (Silva 2004). The activation of STATs by EGFR/Src is mechanistically different from cytokine activated STATs and demonstrates novel tyrosine phosphorylation sites on STAT5b that are phosphorylated in response to EGF but not in response to cytokines (Kloth et al 2002). Furthermore, STAT5b displays a unique nuclear localization (Kabotyanski and Rosen 2003) and transcriptional activation pattern after EGF stimulation but not cytokine stimulation (Guren et al 2003). The EGFR/Src activated STATs are important regulators of cell-cycle progression and apoptosis (Calò et al 2003).

2.2.4.4 PLC-γ

EGF stimulation has significant effects on phospholipid metabolism of a cell. PLC-γ is an enzyme that catalyzes the hydrolysis of phosphatidylinositol (4,5)-biphophate (PIP_2), generating the second messenger diacylglycerol (DAG) and inositol triphosphate (IP_3) (Kamat and Carpenter 1997). IP_3 mediates calcium release affecting calcium–dependent enzymes such as RaI (Hofer et al 1998) and NFκB (Sun and Carpenter 1998). DAG is a coactivator of the serine/threonine kinase PKC that activates multiple signaling components, including the MAPK and JNK pathways (Marais et al 1998, McClellan et al 1999). The activation of PLC-γ by EGF is essential for growth factor induced cell motility (Chen et al 1994).

2.2.4.5 PI3-K

The ErbB receptor activated PI3-K pathway is an important mediator of cell migration, adhesion, proliferation and survival. In the ErbB family ErbB3 is the main recruiter of PI3-K as it harbors at least six binding motifs for the p85 subunit of PI3-K. The interaction with Ras also contributes to the activation of PI3-K and represents another mechanism by which this pathway can be induced (Rodriguez-Viciana et al 1994). The activated PI3-K generates the second messenger, phosphatidylinositol-3,4,5-triphosphate (PIP_3), that recruits the serine-threonine kinases Akt and 3-phosphoinositide-dependent kinase 1 (PDK1) for PDK1 to phosphorylate and activate Akt. The phosphatase and tensin homologue (PTEN) dephosphorylates PIP_3 at the 3' position and acts as an enzymatic antagonist of PI3-K (Zhao and Vogt 2008). Several signaling

cascades originate from activated Akt that regulate for example transcription by inducing degradation of forkhead box O transcription factor (FOXO1) and inactivation of glycogen synthase kinase-3β (GSK3β). Important targets of FOXO1 are the growth attenuating p27 and proapoptotic BAD (Bcl-2 associated death domain) proteins. GSK3β regulates the potentially oncogenic transcription factors Jun and Myc (Zhao and Vogt 2008). Akt signaling generates a positive feedback loop through mammalian target of rapamycin (mTOR) that results in additional Akt activation (Sarbassov et al 2005). Activation of Akt pathway by mutations, overexpression or loss of PTEN is now recognized as a critical mechanism of tumor growth and formation of resistance during drug therapy.

2.2.5 Nuclear signaling of ErbBs

All ErbB receptors are found in the nucleus either as the full-length form or as an intracellular receptor fragment (ICD). The EGF-stimulated nuclear EGFR has been found to directly bind the cyclin D1 promoter and drive cell proliferation demonstrating a transcriptional role for nuclear EGFR (Lin et al 2001). Nuclear ErbB2 binds the cyclooxygenase enzyme COX-2 promoter and stimulates its transcription (Wang et al 2004). Increased COX-2 expression contributes to antiapoptotic, proangiogenic, and metastatic potential in cancer cells. It has also been proposed that EGFR and ErbB4 function as nuclear transporters of receptor-associated phosphorylated transcription factors STAT1, STAT3 and STAT5A (Bild et al 2002, Lo et al 2005, Williams et al 2004). The nuclear ErbB3 has been shown to shuttle between different cellular compartments in response to ligand stimulation (Offterdinger et al 2002). It is not well understood how these full-length ErbB receptors are released from the membrane environment into the cytosol to enter the nucleus. In the case of EGFR, as the nuclear localization is ligand-dependent, the translocation could involve endosomal sorting (Liao and Carpenter 2007).

The mechanism of nuclear localization of proteolytically cleaved soluble receptor ICD fragments is generally well described. ErbB4 is the only member of the ErbB family to undergo a two-step proteolytic cleavage event in response to ligand binding (Zhou and Carpenter 2000) or the activation by protein kinase C by TPA (12-*O*-tetradecanoylphorbol-13-acetate) (Vecchi et al 1996). One assumption for this proteolytic specificity is that ErbB4 JM-a has a longer stalk region in its extracellular domain IV that provides a recognition site for the primary proteolytic enzyme TACE. The proteolytically processed ErbB4 receptor ICD fragment has been shown to translocate to the nucleus and function as a coactivator or corepressor of transcription for different genes (Figure 3). The activities of the extracellular and intracellular cleavage products will be discussed in detail later.

2.2.6 Endocytosis and signal termination

ErbB receptors are predominantly found at the cell surface but are constantly undergoing cycling between the plasma membrane and the endosomal compartment. The EGFR has a metabolic turnover with a half-life of approximately ten hours. Thus, an average receptor will recycle from the endocytic pathway multiple times with a low probability of being degraded (Stoscheck and Carpenter 1984). EGFR has been shown to alter its trafficking in response to ligand binding resulting in accelerated internalization by 5-10 fold and enhanced lysosomal targeting (Baulida et al 1996). The ligand-activated EGFR undergoes internalization via clathrin-coated pits, which is the fastest and highly regulated pathway of internalization of integral membrane proteins. The mechanism by which EGFR is internalized includes phophorylation of the tyrosine residues that bind Grb-2 adaptor protein (Tyr1068 and Tyr1086) (Jiang et al 2003) and several sequence motifs that interact with the clathrin adaptor protein complex 2 (AP-2) (Sorkin and Carpenter 1993, Sorkin et al 1995, Nesterov et al 1995, Sorkin et al 1996, Huang et al 2003). The bound Grb-2 couples Cbl, a RING finger containing E3 ubiquitin ligase, to EGFR and mediates its ubiquitination (Jiang and Sorkin 2003). Several proteins bind the ubiquitinated EGFR, such as Epsin and the ESCRT complexes and mediate its trafficking through the endocytic pathway (Bache et al 2003, Raiborg et al 2008, Roxrud et al 2008).

After internalization, the clathrin-coated vesicles rapidly release their coat and fuse with early endosomes. Early endosomes are highly dynamic and undergo recycling of cargo or fusion with each other to form “sorting” endosomes. The endosomes at this point are mildly acidic (pH 6.0-6.5) leaving the EGF-EGFR complexes dimerized, phosphorylated and associated with Grb-2 and Cbl (Galperin et al 2004, Sorkin et al 1988, Sorkin and Carpenter 1991). The maturation of endosomes to multivesicular bodies (MVB) involves a change in biochemical composition and morphology of early endosomes. The MVBs fuse with primary lysosomal vesicles carrying proteolytic enzymes that lead to rapid proteolysis of intralumenal components of MVBs (Carpenter and Cohen 1976). The internalization of EGFR by clathrin-independent mechanisms have also been observed and involve cholesterol-rich lipid rafts and/or caveolae (Sigismund et al 2005).

Endocytosis of other ErbB receptors has been considered to be less efficient. In fact, ErbB2, when dimerized with EGFR, has been reported to prevent clathrin-mediated endocytosis of EGFR (Haslekås et al 2005, Offterdinger and Bastiaens 2008) or re-route internalized EGFR from degradation to the recycling pathway (Worthylake et al 1999). The half-life of ErbB2 in CHO cells is only three and a half hours compared to ten hours for EGFR (Citri et al 2002). Thus, ErbB2 signaling may be regulated by rapid turnover rather than efficient

internalization. Ligand binding to ErbB3 or ErbB4, however, has been shown to induce receptor ubiquitination and internalization via endosomal sorting. For example, binding of NRG-1 to ErbB3 stabilizes Nrdp1 via the deubiquitination enzyme UBPY/USP8 and leads to enhanced ubiquitination and downregulation of ErbB3 (Cao et al 2007). Also, ErbB4 has been shown to be ubiquitinated by at least three different E3 ubiquitin ligases including Itch, WWP1 and Nedd4 (Feng et al 2009, Omerovic et al 2007, Sundvall et al 2008b, Zeng et al 2009). Sundvall et al demonstrate that the CYT-1 isoform of ErbB4 is more susceptible to internalization, ubiquitination, endocytic targeting and degradation than the CYT-2 isoform as it contains a binding site for the HECT-type ubiquitin ligase Itch (Sundvall et al 2008b). This may differentiate ErbB4 signaling both quantitatively and qualitatively in an isoform dependent manner.

2.2.7 ErbB functions in normal tissues

The importance of ErbB family signaling in normal tissues has been demonstrated by studies with ErbB deficient mice that display a wide range of phenotypes consistent with the roles for the receptors in cell differentiation, proliferation and migration. The EGFR deficient mice die from preimplantation to three weeks postnatally depending on their genetic background and demonstrate defects in various tissues including skin, central nervous system, intestines, lung, liver, kidneys, placenta and palate (Miettinen et al 1995, Miettinen et al 1999, Sibilia and Wagner 1995, Threadgill et al 1995). Strain-independent loss of EGFR gene results in postnatal neurodegeneration within the frontal cortex, olfactory bulbs, and thalamus (Kornblum et al 1998, Sibilia et al 1998). A spontaneous EGFR mutation in the *waved-2* mouse leads to defects in hair follicle and eye development (Luetteke et al 1994). A similar phenotype is observed with a TGF-α deficient mouse strain *waved-1* (Luetteke et al 1993, Mann et al 1993). Mice lacking EGFR ligands EGF, TGF-α or amphiregulin show much less severe phenotypes than EGFR mutant mice and fail to recapitulate in whole the symptoms resulting from the loss of EGFR (Luetteke et al 1999) demonstrating the high degree of redundancy occurring in ErbB signaling. Triple mutant mice deficient in these ligands partially share the EGFR knockout phenotype (Troyer et al 2001).

Mice lacking functional ErbB2 die at mid-gestation due to failure in formation of cardiac trabeculae, a process that involves cardiomyocyte proliferation resulting in thickening of the muscular ventricular wall (Erickson et al 1997, Lee et al 1995). A conditional knockout of the ErbB2 gene in the adult heart leads to postnatal dilated cardiomyopathy and death demonstrating an important role for ErbB2 in the maintenance of the adult heart as well (Crone et al 2002, Ozcelik et al 2002). In fact, cardiac dysfunction is an adverse effect in some patients receiving anticancer drugs targeting ErbB2 (Schneider et al 2001). Rescue of the embryonic lethal ErbB2 deficient mice with myocardium-

restricted expression of wild type ErbB2 results in severe defects in Schwann cell migration with loss of sensory and motor neurons in the peripheral nervous system (Lin et al 2000, Morris et al 1999, Woldeyesus et al 1999). In the spinal cord ErbB2 has a role in the terminal differentiation of oligodendrocyte precursors to mature oligodendrocytes (Park et al 2001). ErbB2 deficient mice also show defects in the cranial sensory ganglia (Lee et al 1995).

ErbB3 null mice have defects in myelination of peripheral nerves due to impaired Schwann cell maturation and have defects in the formation of cerebellum and cranial nerve ganglia (Riethmacher et al 1997). In addition, the ErbB3 null mice reveal a critical step in cardiac development for NRG/ErbB signaling and the mice deficient for ErbB3 die in the uterus at day 13.5. The heart displays severe defects in atrioventricular valve formation that likely contributes to the fetal death (Erickson et al 1997).

Similar to ErbB2 null mice, ErbB4 deficient mice die between embryonic day 10.5-11.5 due to a failure of trabeculation of the ventricular myocardium (Gassmann et al 1995). A conditional disruption of functional ErbB4 within the ventricular myocardium results in dilated cardiomyopathy (García-Rivello et al 2005), a defect also seen in ErbB2 knockout mice. Targeted disruption of NRG-1 leads to defects closely recapitulating the phenotypes of ErbB2 and ErbB4 mutants revealing the importance of the NRG/ErbB2/ErbB4 complex in cardiac development (Meyer and Birchmeier 1995). However, unlike NRG-1 or ErbB2, ErbB4 null mice also have defective innervation of the hindbrain resulting from the loss of an inhibitory effect on axonal path finding by ErbB4 (Gassmann et al 1995). ErbB4 signaling in the mammary epithelium is unique among the ErbB receptors and is required for lobuloalveolar development in the lactating mammary gland (Tidcombe et al 2003). ErbB4 null mammary glands fail to lactate due to decreased STAT5a transcriptional activity (Jones et al 1999, Long et al 2003).

2.3 ErbBs as targets for cancer treatment

2.3.1 ErbBs in cancer

Since the initial discovery in the early 80's that EGFR and ErbB2 share structural similarities with potent animal oncogenes v-ErbB (Downward et al 1984) and c-Neu (Schechter et al 1984), a notable amount of studies have illustrated the importance of altered ErbB signaling in malignant transformation. As a result, ErbB overactivity is often implicated in the pathogenesis of various epithelial and central nervous system malignancies, and ErbB expression is typically related to malignancies with poor clinical outcome (Slamon et al 1987). The recognition of specific gene expression abnormalities and cancer-associated mutations provide both prognostic and predictive information (Linardou et al 2009) and form the basis for therapeutics aiming to specifically

inhibit ErbB activity in cancer (Hynes and Lane 2005). Less is known about the more recently described ErbB family members ErbB3 and ErbB4. ErbB3 is frequently expressed in human tumors of breast, ovarian, lung, colorectal, and prostate cancers (Bobrow et al 1997, Gregory et al 2005, Ljuslinder et al 2009), typically along with the other ErbB family members. In fact, co-expression of other ErbBs with ErbB3 has been shown to promote tumorigenesis in preclinical and clinical settings (Engelman et al 2005, Lodge et al 2003, Tanner et al 2006). ErbB4 is also frequently present in various cancer tissues (Junttila et al 2005, Ljuslinder et al 2009, Rickman et al 2009, Xu et al 2008, Zeng et al 2008), and cancer-associated mutations in the ErbB4 gene have recently been described (Ding et al 2008, Parsons et al 2005, Prickett et al 2009, Soung et al 2006, Tvorogov et al 2009).

2.3.2 Monoclonal antibodies

Monoclonal antibodies have emerged as a new important drug class in the treatment of cancer, multiple sclerosis, chronic inflammatory diseases, cardiovascular medicine and infectious diseases. The strengths of this drug group include that mAbs have a high success rate from the first human experiments to approval (Reichert et al 2005). Much of the development and optimization done for one antibody is applicable to other antibodies decreasing many risks that affect successful drug development. In general, antibodies are well tolerated. However, first dose infusion reactions are commonly observed but are usually manageable. The clinical potential of antibodies can be increased by improving their existing properties by antibody-drug conjugates and Fc optimization to enhance secondary immune functions (Carter 2006). The use of antibodies is, however, limited to targets that are on the surface of the host cells or an invading pathogen. In addition, antibody drugs are expensive due to high cost drug development, manufacturing and the large doses often required for treatment (Scott 2005). The clinically approved antibody therapeutics include a number of unmodified IgG molecules together with a few radio-immunoconjugates and an antibody-drug conjugate (gemtuzumab-ozogamicin).

The genes encoding the constant region of human immunoglobulin produce five different classes of immunoglobulin molecules IgA, IgD, IgE, IgG and IgM that have different roles in mediating host defense and humoral immune responses. The IgD, IgE and IgG are monomers of one immunoglobulin molecule, IgA is a dimer of two Ig molecules and IgM is a pentamer. The IgA and IgG molecules are further divided into different isotypes IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2 based on minor differences in the amino acid sequences of the immunoglobulin heavy chain. The development and use of monoclonal antibodies in cancer therapy has focused on different isotypes of the IgG class.

2.3.2.1 IgG

The IgG molecules of ~150 kDa comprise of a pair of identical heavy and light chains linked to each other by disulfide bonds (Figure 4). The heavy chains are formed from a variable domain (V_H) and three constant (C) domains. The light chain comprises of a variable domain (V_L) but only one constant domain. The high selectivity of antibodies for antigens is mainly mediated by six loops known as the complementary-determining regions (CDR) three of which are present in the V_H and three in the V_L domains. The remaining structures in the V domains are called the framework regions and function mainly to support the CDRs. The Fc region of the antibody structure formed by the last two constant domains of the heavy chain can execute effector functions of antibody-dependent cell cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) and antibody-dependent phagocytosis (ADP). The first two functions require the presence of Fc-region saccharide component to support the interaction between IgG and its receptors FcγRs or complement component 1q (C1q) on effector cells. For example, reducing the fucose content of the Fc region glycosylation increases ADCC activity (Shinkawa et al 2003). However, for some antibody therapeutics avoiding effector functions is desirable to improve treatment and decrease side effects. The terminal half-life of an antibody in plasma is determined by interaction of its Fc region with the salvage receptor FcRn. The IgG binds the FcRn receptor in endosomes after fluid phase uptake and is recycled back to the cell surface for release (Ober et al 2004a, Ober et al 2004b). By tailoring this interaction, the plasma half-life of an antibody can be prolonged to improve localization to the target, increase efficacy and reduce dose or frequency of administration or be shortened to reduce whole-body exposure and improve the target-to-non-target ratio.

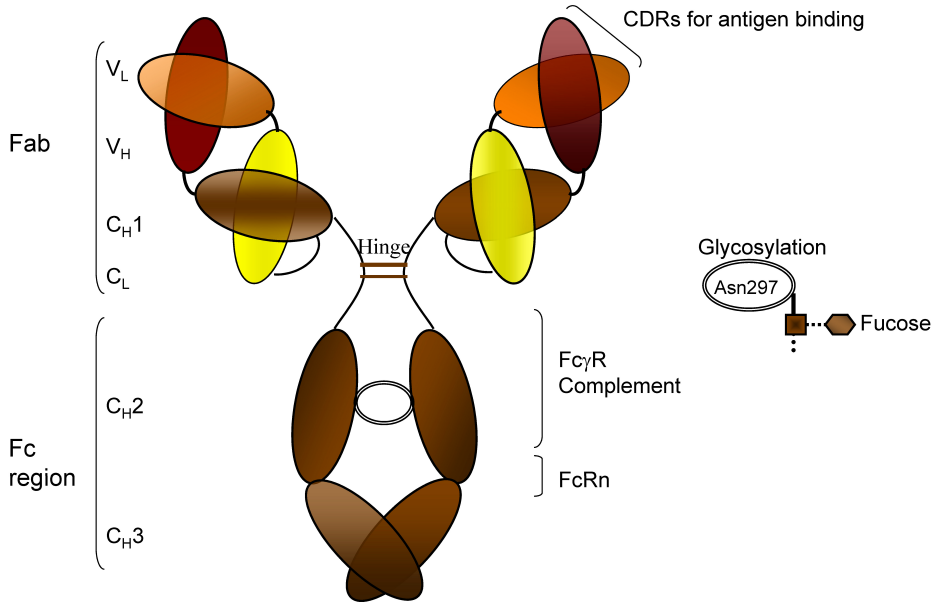

Figure 4. Structure of a monoclonal antibody of the IgG class.
The IgG structure comprises of two heavy chains and two light chains linked to each other by disulfide bonds. Breakage of these bonds by protease activity forms two fragments called the Fab fragment and the Fc region. The Fab fragment contains the variable antibody binding regions of both the heavy (V_H) and light (V_L) chains and the first constant (C_H1) region of the heavy chain and the constant (C_L) region of the light chain. The distal parts of the variable domains form loop structures known as the complementary determining regions (CDRs) that mainly mediate binding to the antigen. The Fc region formed by the two constant (C_H2 and C_H3) regions of the heavy chains contain glycosylation of an asparagine (Asn) residue for mediating effector cell functions via FcγR and complement receptors. The effector functions are crucially influenced by the glycosylaton occurring at Asn297. The Fc region also binds the salvage receptor FcRn and influences the cellular uptake and degradation of the IgG via endosomal sorting.

The amino acid differences in the Fc region of IgG isotypes result in differential binding to FcγRs and FcRn, which discriminates the IgG isotypes in mediating effector functions and *in vivo* half-life (Table 1) (Salfeld 2007). The IgG2 isotype can form covalent dimers *in vivo* between identical or different IgG molecules with intermolecule disulfide bonds (Yoo et al 2003). This results in a tetravalent IgG molecule that may present different pharmacokinetics or targeting of the dimer. Although the IgG3 subtype is as good in mediating effector functions as IgG1, it has not been used for therapeutic antibody development because of its relatively short half-life, susceptibility to proteolysis by its long hinge region and extensive allotypic polymorphism (Carter 2006). The IgG4 isotype can exchange vertically half of its IgG molecule by its hinge

region flexibility in a dynamic process termed Fab-arm exchange creating monospecific or bispecific IgG4s (van der Neut Kolfschoten et al 2007). It is proposed by van der Neut Kolfschoten et al that the Fab-arm exchange of IgG4 *in vivo* has protective value in immune responses to self-antigens found frequently in autoimmune disorders.

Table 1. Different properties of the IgG isotypes

Isotype	**IgG1**	**IgG2**	**IgG3**	**IgG4**
Structure and valency *in vivo*	Monomer bivalent	Dimer tetravalent	Monomer bivalent	Half-monomer monovalent
Half-life (d)	36.3 ± 9.2	37.1 ± 13.9	28.6 ± 10.4	15.6 ± 4.5
Allotypes	4	1	13	0
FcRn	+	+	+	+
Hinge length (aa)	15	12	62	12
ADCC (FcγRI)	+++	-	+++	++
ADCC (FcγRII)	+	±	+	?
ADCC (FcγRIIIa/b)	+	-	+	±
CDC (C1q)	++	-	+++	-

Ig immunoglobulin, *d* days, *allotype* genetic variability in the constant domains of immunoglobulin isotype, *FcRn* salvage receptor, *ADCC/CDC* immune effector functions

2.3.2.2 Development of mAbs

The first developed and most widely used method for generating monoclonal antibodies was the hybridoma fusion technique developed by Köhler and Milstein in 1975 (Köhler and Milstein 1975). This technique introduced the use of mouse hybridomas generated from the stable fusion of immortalized myeloma cells with B cells from immunized mice. These mouse antibodies have been successfully used in research since then. However, the direct application of mouse monoclonal antibodies (mAb) in the clinic is nearly always unsuccessful due to their high immunogenicity and weak interaction with human FcγR, complement and FcRn receptors (Ober et al 2001, Presta et al 2002). The limitations are largely overcome by mouse monoclonal antibody chimerization and humanization (Gonzales et al 2005, Kipriyanov and Le Gall 2004). Chimerization involves joining the variable (V) antigen binding domains of a mouse monoclonal antibody to the constant domains of a human antibody (Boulianne et al 1984, Morrison et al 1984). The simplest humanization strategy involves the transfer of the CDRs from a mouse antibody to a human IgG (Jones et al 1986, Riechmann et al 1988). For high binding affinity, additional transfer of one or more framework region residues from the parent mouse antibody is

generally required (Gonzales et al 2005). A growing number of antibodies entering clinical trials are completely human and are generated from phage-display technique or transgenic mice that express human immunoglobulin genes. The phage-display is a technology for displaying a protein, in this case an antibody fragment, on the surface of a bacteriophage that contains the genes encoding the fragment. The displayed fragments are usually single-chain V-domain antibody fragments (scFv) or Fab fragments of an antibody molecule (Marks et al 1991, McCafferty et al 1990). The scFvs are comprised of the variable domains of the immunoglobulin heavy and light chains joined together by a short peptide linker (~15 aa). A combinatorial library of up to 10^{11} genes cloned for display is used to screen the best binding properties for a selected target. The scFv and Fab molecules alone have a very short half-life in plasma and are inefficient to support effector functions. Thus, the reformatting of these molecules to full IgGs is required (Jostock et al 2004, Sarantopoulos et al 1994). Immunizing mice transgenic for human immunoglobulin genes can also generate fully human monoclonal antibodies (Green et al 1994, Lonberg et al 1994). The direct production of human IgG from the hybridomas removes the need for antibody engineering and supports direct preclinical and clinical testing.

2.3.2.3 mAbs targeting EGFR

Antibodies targeting the extracellular domains of ErbB receptors have been extensively studied in drug development (Table 2). The research carried out with these antibodies has also provided a mechanistic understanding on ErbB receptor structure and functions (Schmitz and Ferguson 2009). The EGFR targeting monoclonal antibodies that are either approved for clinical use (cetuximab and panitumumab) or are in late clinical trials (zalutumumab and nimotuzumab) bind residues on the ligand-binding domain III (Bleeker et al 2004, Crombet-Ramos et al 2002, Jakobovits et al 2007, Li et al 2005, Schmiedel et al 2008) and compete for ligand-binding and consequent EGFR activation (Bleeker et al 2004, Crombet-Ramos et al 2002, Jakobovits et al 2007, Li et al 2005). Matuzumab also binds domain III but inhibits EGFR activity by sterically preventing conformational changes required for the formation of receptor dimers (Schmiedel et al 2008). A similar mechanism of action has also been reported for cetuximab (Li et al 2005), and zalutumumab (Bleeker et al 2004, Lammerts van Bueren et al 2008) that limits both intra- and intermolecular flexibility of the EGFR extracellular domain. Panitumumab, unlike the other EGFR antibodies mentioned, belongs to the IgG2 subtype and is less effective in mediating ADCC (Cohenuram and Saif 2007). However, it has a relatively long half-life and can be administered less frequently than the typical weekly dose used for most protocols (Jakobovits et al 2007). The fully human antibodies panitumumab and zalutumumab are expected not to evoke human anti-mouse antibody (HAMA) production as compared to the chimeric

or humanized antibodies that include short mouse-specific sequences (Cohenuram and Saif 2007, Ruuls et al 2008).

Table 2. Therapeutic EGFR and ErbB2 antibodies

Antibody	Mechanism of action	Indications
EGFR		
cetuximab/ **Erbitux** *chimeric*	Blocks ligand binding and prevents conformational changes required for dimerization.	KRASwt mCRC in combination with chemotherapy for first-line treatment or as a single agent for second-line treatment. HNSCC in combination with radiotherapy for first-line treatment or in combination with chemotherapy for second-line treatment.
panitumumab/ **Vectibix** *fully human*	Blocks ligand binding and receptor activation. Subtype IgG2.	KRASwt mCRC as a single agent for second-line treatment
EMD 72000/ matuzumab *humanized*	Prevents conformational changes required for dimerization.	No longer under clinical trials due to disappointing results in mCRC, lung, gastric and esophageal cancers.
HuMax-EGFr/ zalutumumab *fully human*	Blocks ligand binding and restricts intra- and intermolecular flexibility.	FDA fast track status for HNSCC after failure of conventional treatments. Ongoing phase III trials for HNSCC in combination with chemo and radiotherapy.
nimotuzumab/ TheraCim *humanized*	Blocks ligand binding.	Limited national approval for HNSCC and glioma in combination with conventional therapy for first-line or adjuvant treatment. Several ongoing phase III trials for various cancers.
ErbB2		
trastuzumab/ **Herceptin** *humanized*	Blocks ectodomain shedding. Disrupts ErbB2/ErbB3 activation.	*ErbB2+ metastatic breast cancer as:* 1° single agent for second-line treatment, 2° in combination with paclitaxel, 3° docetaxel or 4° aromatase inhibitors for first-line treatment. *ErbB2+ operable breast cancer as* adjuvant treatment after or in combination with adjuvant chemotherapy.
pertuzumab/ Omnitarg *humanized*	Prevents heterodimerization.	Phase III trial in combination with trastuzumab for first-line treatment, ErbB2+ metastatic breast cancer

mCRC metastatic colorectal carcinoma, *wt* wild type, *HNSCC* head and neck squamous cell carcinoma, *NSCLC* non-small cell lung cancer

2.3.2.4 mAbs targeting ErbB2

The ErbB2 targeting antibodies trastuzumab and pertuzumab bind exposed structural domains on ErbB2 that are important for dimerization activities of the extended ligandless receptor (Figure 1; Table 2). Trastuzumab is a humanized antibody derived from a murine monoclonal antibody clone 4D5 (muMab4D5). It has been shown to inhibit ErbB2-dependent tumorigenesis by several mechanisms. As a humanized IgG1 subtype antibody, trastuzumab binds the Fcγ receptor III and mediates ADCC (Clynes et al 2000). Initially trastuzumab was also proposed to decrease ErbB2 phosphorylation (Sarup et al 1991) and increase ErbB2 endocytosis and degradation (Hudziak et al 1989). However, as concluded by Austin et al., trastuzumab does not seem to affect the turnover rate of ErbB2 (Austin et al 2004) nor is it effective in blocking dimerization with ligand-activated EGFR or ErbB3 (Agus et al 2002). Instead, trastuzumab treatment leads to G_1-S cell cycle arrest (Yakes et al 2002) and leads to downregulation of Akt activity (Lane et al 2000). Trastuzumab binds to the extracellular domain IV (Cho et al 2003) and has been suggested to inhibit ErbB2 ectodomain shedding by ADAM10, as well as subsequent ligand-independent activation of the truncated membrane-anchored receptor fragment (Liu et al 2006, Molina et al 2001). In fact, Junttila et al have shown that trastuzumab disrupts ligand-independent constitutive ErbB2/ErbB3 complexes and blocks downstream PI3-K/Akt signaling decreasing the transformation potential of ErbB2 (Junttila et al 2009). This is consistent with the observation that ErbB3 has been recognized as an important mediator of ErbB2 survival signals (Holbro et al 2003).

Trastuzumab has shown efficacy for patients with amplification of the ErbB2 gene in breast cancer tissue detected by immunohistochemistry (IHC) or fluorescence in situ hybridization (FISH) (Wolff et al 2007). The current dose (4 mg/kg, followed by 2 mg/kg) and schedule (typically once weekly) of trastuzumab was established in a prospective randomized study of 114 metastatic breast cancer patients with response rates of 35% with trastuzumab as a single agent in first-line treatment (Vogel et al 2002). A pivotal randomized clinical trial of trastuzumab in combination with chemotherapy of 469 previously untreated, ErbB2-positive, metastatic breast cancer patients showed significant increase in the median time to disease progression and overall survival (Slamon et al 2001) (Table 3). In this study the cardiotoxicity of trastuzumab became evident as a total of 27% of the patients treated with trastuzumab and anthracyclines experienced cardiac dysfunction (Slamon et al 2001). It has been suggested that the reason for frequently observed cardiotoxicity with this drug combination is a result from inhibition of ErbB2 signaling in heart tissue where ErbB2 is needed for repair of anthracycline-induced cardiac myocyte damage (Crone et al 2002, Ozcelik et al 2002). The carditoxic effects of trastuzumab are less severe and readily reversible compared

to the effects of anthracyclines (Perez and Rodeheffer 2004, Seidman et al 2002).

The promising results of trastuzumab treatment for metastatic breast cancer led to the investigation of this drug also in the adjuvant (post-surgical) setting. In two North American studies eligible patients with early-stage, lymph node and ErbB2-positive breast cancer received doxorubicin and cyclophosphamide followed by paclitaxel with or without trastuzumab for one year (Romond et al 2005) (Table 3). Adding trastuzumab for adjuvant treatment significantly increased disease-free survival and overall survival. Also, the results in the Herceptin Adjuvant (HERA) trial with more than 5000 patients showed a significant reduction in the risk of recurrence with trastuzumab. The estimated two-year disease-free survival was 85.8% versus 77.4% (Piccart-Gebhart et al 2005). In the FinHer trial adjuvant trastuzumab given concominantly with chemotherapy as nine weekly doses was effective in preventing breast cancer recurrence. Importantly, trastuzumab was well tolerated and the left ventricular ejection fractions were maintained during the three-year follow-up (Joensuu et al 2006).

Table 3. Major clinical trials with trastuzumab

Study (n)	HR for recurrence/death	*P* - value	Reference
Trastuzumab for *ErbB2 positive metastatic breast cancer*			
(n=469)	One-year follow-up		(Slamon et al 2001)
In combination with chemotherapy for first-line treatment.	DFS: 0.51 (0.41-0.63) OS: 0.80 (0.64-1.00)	P < 0.001 P = 0.046	
Trastuzumab for *ErbB2 positive operable breast cancer*			
National surgical adjuvant breast and bowel project B-31 and North Central Cancer treatment group N9831 (n=3351)	Three-year follow-up		(Romond et al 2005)
In combination with adjuvant chemotherapy for 1 year	DFS: 0.48 (0.39-0.59) OS: 0.67 (0.48-0.93)	P < 0.0001 P = 0.015	
Herceptin adjuvant trial (HERA) (n=5090)	One-year follow-up		(Piccart-Gebhart et al 2005)
As a single agent after adjuvant therapy for 1-2 years	DFS: 0.54 (0.43-0.67) OS not significant	P < 0.0001	
FinHer trial (n=1010)	Three-year follow-up		(Joensuu et al 2006)
In combination with adjuvant chemotherapy (nine weekly doses)	DFS: 0.42 (0.21-0.83) OS: not significant	P = 0.01	

HR hazard ratio, *DFS* disease-free survival, *OS* overall survival

A high proportion of trastuzumab-treated patients end up developing symptomatic brain metastases (Torresi 2008). One reason for this phenomenon might be that trastuzumab does not cross the blood-brain barrier (Stemmler et al 2007) thus leaving the central nervous system untreatable. The development of resistance to trastuzumab is also frequent. Resistance is developed within one year in the majority of patients with initial response to trastuzumab (Nielsen et al 2009). Many mechanisms have been proposed to account for trastuzumab resistance and involve altered receptor-antibody interaction, increased signaling from other receptors and constitutive activation of downstream effectors. The membrane-associated glycoprotein mucin-4 (MUC4) interacts directly with ErbB2 and might prevent trastuzumab binding to ErbB2 (Price-Schiavi et al 2002). *In vitro*, MUC4 expression is associated with resistance of breast cancer cells and inversely correlates to trastuzumab affinity for ErbB2 (Nagy et al 2005). Also, increased ErbB2 shedding in cancer tissues leads to the formation of a truncated, constitutively active p95 ErbB2 receptor fragment that escapes any targeting to the extracellular domain (Molina et al 2002) promoting resistance to trastuzumab treatment in patients (Scaltriti et al 2007). It has also been hypothesized that the remaining extracellular domain of the soluble ErbB2 increases resistance to trastuzumab by neutralizing its effects.

A significant level of cross talk occurs within the ErbB family with compensatory effects for abolished ErbB family members (Bender and Nahta 2008, Brockhoff et al 2007, Diermeier et al 2005). The increased level of ErbB ligands and co-expression of ErbB3 has been reported to increase resistance for trastuzumab (Robinson et al 2006, Valabrega et al 2005). Importantly, decreased levels of PTEN block trastuzumab-mediated growth inhibition in ErbB2 overexpressing breast cancer cells (Yakes et al 2002) and predict resistance in patients (Nagata et al 2004). These observations indicate that the PI3-K pathway is a crucial mediator of trastuzumab resistance (Berns et al 2007). However, trastuzumab treatment has also been found to increase Notch activity, possibly compensating for the inhibiton of ErbB2, suggesting that Notch may contribute to trastuzumab resistance (Osipo et al 2008).

Pertuzumab binds epitopes on the exposed dimerization motif on domain II and inhibits ErbB2 dimerization with other ErbB family members (Franklin et al 2004). A recent report by Cai et al. (Cai et al 2008) demonstrates the diversity in the formation of different heterodimers and shows that pertuzumab preferentially inhibits ErbB2 dimerization with ErbB3 rather than EGFR. Pertuzumab, in contrast to trastuzumab, is also functional in tumors with low ErbB2 expression. However, it has shown limited activity as a single agent in ovarian (Gordon et al 2006), prostate (Agus et al 2007) and NSCLC (Herbst et al 2007) and has been investigated in clinical setting in combination with trastuzumab and chemotherapy with promising results (Portera et al 2008, Walshe et al 2006).

2.3.3 Tyrosine kinase inhibitors targeting ErbB receptors

Another approach to target overactive ErbB signaling has led to the development of small-molecule tyrosine kinase inhibitors (TKI) that compete with or block ATP binding to the receptor kinase enzyme. The benefit of these drugs as compared to monoclonal antibodies is that they are orally active and membrane-permeable. Because of their small size, they are likely to pass the blood-brain barrier. TKIs are generally thought to be less specific than antibodies with some risk of toxicity. However, in some cases lower specificity might be clinically advantageous. Imatinib (Glivec) targets tyrosine kinases such as ABL, KIT and PDGFR. Thus it has been approved for the treatment of chronic myeloid leukemia caused by the BCR-ABL fusion, chronic eosinophilic leukemia caused by a FIP1L1-PDGFRα fusion and gastrointestinal stromal tumors (GISTs), caused by mutations of KIT or PDGFRα (Druker 2008).

Two reversible inhibitors gefitinib (Iressa) and erlotinib (Tarceva) targeting EGFR have been approved for the treatment of locally advanced or metastatic NSCLC and erlotinib also for pancreatic cancer in combination with gemcitabine. The efficacy of gefitinib and erlotinib in NSCLC seems to be limited to a subpopulation of patients harboring EGFR mutations (Lynch et al 2004, Pérez-Soler et al 2004), and patients of East Asian descent (Fukuoka et al 2003). However, Tsao et al. have shown that the presence of EGFR mutations in NSCLC may increase responsiveness to erlotinib, but do not predict a survival benefit (Tsao et al 2005).

A recent clinical study with a dual EGFR and ErbB2 TKI, lapatinib (Tyverb), led to approval of its use for advanced ErbB2-positive breast cancer as it was effective in increasing progression-free survival in combination with capecitabine in patients with advanced ErbB2-positive breast cancer who had previously failed to respond to therapy with anthracyclines, taxanes and trastuzumab (Geyer et al 2006). In this study it was also observed that the central nervous system metastases occurred less frequently in lapatinib treated patients. Indeed, there is sufficient evidence to support that lapatinib crosses the blood-brain barrier, providing a basis for testing lapatinib in patients with brain metastases (Lin et al 2008). Labatinib, as well as, several other reversible and irreversible dual (neratinib, HKI-272; BIBW-2992) and pan-ErbB TKIs (canertinib, CI-1033; JNJ-28871063) are in clinical trials for NSCLC, head and neck cancer, CRC, glioma, prostate and breast cancer (Baselga and Swain 2009).

Resistance for gefitinib and erlotinib develop within a year of treatment. The suggested mechanisms involve a mutation in the kinase domain that increases EGFR affinity for the competing physiologic substrate, ATP rather than the TKIs (Yun et al 2008). The irreversible TKIs have been reported to partly

overcome resistance in contrast to the reversible TKIs (Engelman et al 2007a) by covalently binding Cys-797 on EGFR and have a prolonged effect in preventing signaling from the receptor (Kwak et al 2005). Resistance, however, may also occur through focal MET amplification that activates the PI3-K pathway via ErbB3 (Engelman et al 2007b). Also, KRAS mutation status in NSCLC is an indicator of resistance to the EGFR TKIs (Massarelli et al 2007).

2.3.4 Cancer-associated somatic mutations of ErbB receptors

Cancer-associated somatic mutations of EGFR are mainly found in a subset of NSCLC patients, more frequently in individuals of East Asian descent (Sharma et al 2007). The tyrosine kinase domain mutations of EGFR, most commonly an in-frame deletion ΔE746-A750 (45%) or L858R (45-50%) in NSCLC, cause a gain-of-function phenotype that results from enhanced kinase activity of the receptor (Paez et al 2004). The mutations also confer sensitivity to EGFR tyrosine kinase inhibitors gefitinib and erlotinib providing a predictive marker for response (Jackman et al 2006). Another clinically relevant mutation of EGFR is a secondary mutation occurring in T790M that promotes resistance to the tyrosine kinase inhibitors and is detected in ~50% of TKI-resistant NSCLC patients that initially responded to gefitinib or erlotinib (Pao et al 2005). This mutation has been thought to weaken the interaction of the inhibitor with its target by affecting the "gatekeeper" residue in the catalytic domain of the kinase, although this mutation still remains sensitive to irreversible TKIs (Engelman et al 2007a). However, a recent report by Yun et al. show that increased ATP affinity caused by T790M is the primary mechanism by which the mutation confers drug resistance (Yun et al 2008).

Mutations of the EGFR kinase domain in CRC, gliomas, HNSCC, esophageal and pancreatic adenocarcinomas have also been reported (Barber et al 2004, Kwak et al 2006, Lee et al 2005). A deletion mutant of EGFR (EGFRvIII) that lacks all of extracellular domain I and a part of domain II is frequently found in glioblastomas causing ligand-independent overactive signaling of EGFR (Pedersen et al 2001). For ErbB2, kinase domain mutations have been reported in 2% of NSCLC, 5% of gastric, 2.9% colorectal and 4.3% of breast carcinomas (Lee et al 2006a, Lee et al 2006b, Shigematsu et al 2005) at residues important for ErbB2 kinase activity. Somatic mutations of ErbB4 have also been reported and will be discussed in detail later. However, it seems that no significant mutations of ErbB3 have been reported.

2.4 ErbB4

ErbB4 is expressed as four alternatively spliced and functionally disctinct isoforms (Figure 5) (Junttila et al 2003). Two of the isoforms differ in the intracellular cytoplasmic domain (isoforms CYT-1 and CYT-2) and two in the extracellular juxtamembrane region (isoforms JM-a and JM-b).

2.4.1 Intracellular cytosolic (CYT) isoforms

The CYT-1 isoform contains a 16 amino acid sequence transcribed from exon 26 that is capable of directly coupling to PI3-K via a *YTPM* sequence (Elenius et al 1999). CYT-1 mediates PI3-K-regulated survival and migration in vitro (Kainulainen et al 2000). The CYT-2 isoform lacks this insertion and cannot directly couple to the PI3-K pathway. The stretch of unique amino acids within the CYT-1 isoform-specific sequence also includes an interaction motif (*PPAY*) for the Itch, WWPI and Nedd4 E3 ubiquitin ligases that regulate ErbB4 monoubiquitination, endocytosis and degradation in an isoform-specific manner (Feng et al 2009, Omerovic et al 2007, Sundvall et al 2008b, Zeng et al 2009).

2.4.2 Extracellular juxtamembrane (JM) isoforms

The extracellular juxtamembrane isoforms are alternatively spliced to include either sequences from the 23 amino acid long exon 16 (JM-a) or from the 13 amino acid long exon 15 (JM-b). The longer JM-a isoform can be cleaved by TACE (Rio et al 2000) whereas the JM-b isoform is proteinase-resistant (Elenius et al 1997a). The cleavage site is located between His651 and Ser652 within the JM-a-specific extracellular juxtamembrane region (Cheng et al 2003). Thus, TACE activity generates a shed soluble extracellular domain of approximately 120 kDa, as well as a membrane-bound truncated receptor fragment of 80 kDa (called the m80). Extracellular cleavage by TACE can subsequently trigger a second cleavage of ErbB4 in the transmembrane region by γ-secretase activity (Lee et al 2002a, Ni et al 2001). This process of two consecutive proteolytic events is also known as regulated intramembrane proteolysis (RIP) (Heldin and Ericsson 2001).

2.4.3 Signaling of the RIP-released ErbB4 ICD

γ-Secretase activity releases a soluble intracellular domain (s80), which can translocate into the nucleus and function as a transcriptional coactivator or corepressor. Transcription factors that have been reported to associate with the ICD of ErbB4 include STAT5 (Williams et al 2004), yes-associated protein (YAP) (Komuro et al 2003), ETO2 (Linggi and Carpenter 2006), ER (Zhu et al 2006) and the TAB2-NCoR complex (Sardi et al 2006). Interaction of nuclear ICD with TAB2-CoR regulates astrogenesis in the developing mouse brain *in vivo* (Sardi et al 2006). The released ErbB4 ICD may also promote apoptosis either by utilizing a BH3-like proapoptotic domain within ErbB4 itself (Naresh et al 2006) or by associating with the p53 regulator Mdm2 (Arasada and Carpenter 2005). Moreover, the cleavable JM-a CYT-2, but not non-cleavable JM-b CYT-2, promotes growth of 32D myeloid cell and MCF-7 breast cancer cell transfectants (Määttä et al 2006), and induces tubulogenesis of MDCK kidney epithelial cells (Zeng et al 2007). The ErbB4 ICD is negatively regulated by the tumor suppressor WW domain-containing oxidoreductase (Wwox) that

prevents nuclear translocation of ICD and promotes accumulation of full-length ErbB4 at the membrane of breast cancer cells (Aqeilan et al 2005, Aqeilan et al 2007).

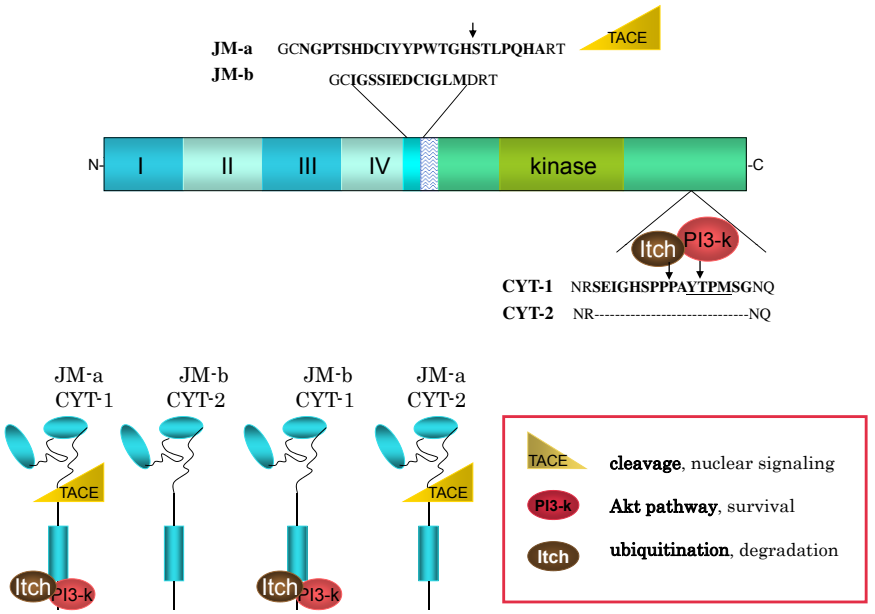

Figure 5. Distinct biological activities of the ErbB4 isoforms
The ErbB4 gene is transcribed into four different isoforms by alternative splicing. The juxtamembrane isoforms are formed either from exon 16 encoding 23 amino acids (JM-a) or exon 15 encoding 13 amino acids (JM-b). The JM-a includes a TACE cleavage site that results in proteolytic processing by TACE, and subsequently by γ-secretase activity, and nuclear signaling by the soluble intracellular receptor fragment. The C-terminal isoforms either include sequence from exon 26 encoding 16 amino acids (CYT-1) or not (CYT-2). The 16 amino acid stretch includes binding motifs for PI3-K and the ubiquitin ligase Itch. TM indicates the transmembrane domain. aa, amino acid.

2.4.4 Expression of the different ErbB4 isoforms

The ErbB4 isoforms, in addition to structural and functional differences, also vary in distribution in both normal and malignant tissues. For example, JM-a is the only JM isoform expressed in kidney, salivary gland, trachea, thyroid gland, prostate and mammary gland (Junttila et al 2005). Brain, in turn, has been shown to express both juxtamembrane isoforms and the heart expresses predominantly the JM-b isoform (Junttila et al 2005). The CYT isoforms are both present when ErbB4 is expressed in tissues. Few reports have so far addressed the expression of different isoforms in cancer tissues. Typically the cleavable JM-a isoform seems to be predominantly present in cancerous tissues,

whereas both CYT isoforms are again present when ErbB4 is expressed (Junttila et al 2005, Zeng et al 2008).

2.4.5 ErbB4 in cancer

ErbB4 is expressed in several cancer types and is found to associate with distinct disease characteristics and survival (Table 4).

2.4.5.1 Breast cancer

Most reports of ErbB4 expression in breast cancer associate ErbB4 with ER- and PgR-positivity, ErbB2 receptor-negativity, well-differentiated phenotype, and favorable outcome in both primary and invasive tumors (Abd El-Rehim et al 2004, Bacus et al 1996, Barnes et al 2005, Haas et al 2009, Kew et al 2000, Knowlden et al 1998, Koutras et al 2008, Sassen et al 2008, Srinivasan et al 2000, Suo et al 2002, Witton et al 2003). Also, loss of ErbB4 expression has been associated with resistance to the ER antagonists (Frogne et al 2009, Guler et al 2007, Naresh et al 2008). Biéche et al. however, report both under- as well as overexpression of ErbB4 mRNA in breast cancer and demonstrate that overexpression of ErbB4 mRNA is associated with ER-positivity but also with shorter relapse-free survival (Bièche et al 2003). Lodge et al. also show that ErbB4 overexpression correlates with decreased survival in tumors with lymph node involvement (Lodge et al 2003). The immunohistochemically detected ErbB4 staining pattern either in the nucleus or in other cellular compartments may add prognostic value for determining the significance of ErbB4 activities in breast cancer. Within the subset of ErbB4-positive breast cancer, nuclear ErbB4 immunoreactivity associates with worse survival when compared with ErbB4 staining on the cell membrane (Junttila et al 2005). Membranous ErbB4 staining associates with favorable survival when compared with tumors without such ErbB4 expression (Aqeilan et al 2007).

2.4.5.2 CNS malignancies

ErbB4 function is required for normal development of the central nervous system (Gassmann et al 1995, Sardi et al 2006) and its abnormal activity is implicated in several pathologies of the brain including malignancies (Chaudhury et al 2003, Law et al 2007). Overexpression of ErbB4 in CNS malignancies typically associates with poor prognosis (Gilbertson et al 1997, Gilbertson et al 2002, Torp et al 2007, Zeng et al 2008). Ependymomas also show an increased proliferation index (Ki-67) when ErbB4 is expressed together with ErbB2 (Gilbertson et al 2002). Medulloblastoma, ependymoma and pilocytic astrocytomas all seem to demonstrate selectively elevated levels of mRNA encoding the cleavable JM-a isoform (Gilbertson et al 2002, Zeng et al 2008). A PCR-based analysis has also indicated a shift in the CYT-1/CYT-2

ratio to favor the CYT-1 isoform in the more aggressive histological subtypes of medulloblastoma (Ferretti et al 2006).

2.4.5.3 Colorectal cancer

In colorectal carcinomas Lee et al report an increase of ErbB4 expression in late-stage tumors and an association of ErbB4/ErbB2 co-expression with short overall survival (Lee et al 2002b). In addition, membranous ErbB4 expression, but not cytoplasmic expression, associates with lymph node involvement (Kountourakis et al 2006), and is an independent prognostic factor of recurrence (Baiocchi et al 2009). Nuclear ErbB4 immunoreactivity has not been evaluated in any of the CRC studies.

2.4.5.4 Squamous cell carcinomas

The squamous cell carcinomas (SCC) of the esophagus and head and neck all show overexpression of ErbB4. Membranous ErbB4 staining in esophageal SCC associates with favorable survival, and nuclear immunoreactivity is an independent marker of poor survival (Xu et al 2008). Xia et al have demonstrated that overexpression of ErbB4 significantly associates with distant metastasis and decreased overall survival in oral SCCs (Xia et al 1999).

2.4.5.5 Other cancer types

In NSCLC ErbB4 expression (25% of patients) does not correlate with survival but negative staining for ErbB4 significantly favors response to chemotherapy (Merimsky et al 2001). Another report demonstrates ErbB4 expression in 48% of patients with osteosarcoma, and association of ErbB4 expression with poor response to chemotherapy as well as short disease-free survival (Merimsky et al 2003). Also in limb soft-tissue sarcoma, increased ErbB4 expression is common in cases with no response to chemotherapy and associated with shorter disease-free survival (Merimsky et al 2002). In bladder cancer ErbB4 expression seems to associate with favorable outcome: Low ErbB4 expression associates with low grade of differentiation, invasiveness and short survival (Kassouf et al 2008), whereas high ErbB4 expression associates with favorable prognosis (Memon et al 2004).

Table 4. Association of ErbB4 expression with disease characteristics and survival

Favorable prognosis			*Poor prognosis*		
Breast	Increased DFS	(Koutras et al 2008)	Breast	Decreased survival when nuclear	(Tovey et al 2006)
Breast	Tamoxifene-sensitivity	(Guler et al 2007)	Breast	Decreased survival when nuclear	(Junttila et al 2005)
Breast	Nonrecurrence and ER+	(Barnes et al 2005)	Breast	Decreased RFS in ER+ tumors	(Bièche et al 2003)
Breast	Increased DFS	(Abd El-Rehim et al 2004)	Breast	Decreased survival when cytoplasmic	(Lodge et al 2003)
Breast	ER+ and increased survival	(Witton et al 2003)	MB	CYT-1/CYT-2 ratio associates with more aggressive disease	(Ferretti et al 2006)
Breast	Increased DFS in ErbB2+ tumors	(Suo et al 2002)	MB	Decreased survival when co-expressed with ErbB2	(Gilbertson et al 1997)
Breast	Low grade when nuclear	(Srinivasan et al 2000)	Epen-dymoma	Enhanced proliferation when co-expressed with ErbB2	(Gilbertson et al 2002)
Breast	Differentiated phenotype	(Kew et al 2000)	CRC	Recurrence	(Baiocchi et al 2009)
Breast	ER+, PgR+, ErbB2-	(Bacus et al 1996)	CRC	LN+	(Kountourakis et al 2006)
Bladder	Downregulation associates with decreased survival	(Kassouf et al 2008)	CRC	Decreased OS when co-expressed with ErbB2	(Lee et al 2002b)
Bladder	Increased survival in tumors with EGFR or ErbB2	(Memon et al 2004)	SCC	Reduced survival when nuclear	(Xu et al 2008)
Thyroid	Small tumor size	(Wiseman et al 2008)	HNSCC	Metastasis and decreased survival	(Xia et al 1999)
			NSCLC	Resistance to chemotherapy	(Merimsky et al 2001)
			Sarcoma (osteo)	Poor response to chemotherapy and short DFS	(Merimsky et al 2003)
			Sarcoma (soft-tissue)	Resistance to chemotherapy and short DFS	(Merimsky et al 2002)

DFS disease-free survival, *ER* estrogen receptor, *RFS* relapse-free survival, *LN* lymph node, *OS* overall survival, *PgR* progesterone receptor, *MB* medulloblastoma, *HNSCC* head and neck squamous cell carcinoma, *NSCLC* non-small cell lung cancer

2.4.5.6 Cancer-associated somatic mutations of ErbB4

Mutations in the ErbB4 tyrosine kinase domain are found in melanoma (Prickett et al 2009), NSCLC (Ding et al 2008, Soung et al 2006), as well as in colorectal, gastric and breast cancer (Soung et al 2006) (Figure 6). The reported frequency of ErbB4 mutations is 2.3% - 4.8% in NSCLC, 2.9% in CRC, 1.7% in gastric, and 1.1% in breast cancer (Ding et al 2008, Soung et al 2006). A recent report by Prickett et al shows a frequency of 19% of activating somatic mutations of ErbB4 in metastatic melanoma patients (Prickett et al 2009). These mutations are scattered throughout the ErbB4 gene and seven of them including missense mutations of the extracellular domain II and IV and the kinase domain cause increased kinase activity and transforming potential (Prickett et al 2009). Interestingly the increased kinase activity of the mutant ErbB4 receptors also increases sensitivity to the pan-ErbB TKI lapatinib. Lapatinib promotes apoptosis in mutant ErbB4 melanoma cell lines suggesting that these mutants confer "oncogene addiction" (Weinstein 2002).[1] In contrast to the findings of Prickett et al. two other mutations (D861Y, G802dup) found in CRC and NSCLC, respectively (Soung et al 2006), cause a significant loss of ErbB4 tyrosine kinase activity indicating a loss-of-function phenotype for ErbB4 in cancer (Tvorogov et al 2009). However, the attenuated kinase activity only kills the intrinsic kinase activity of ErbB4 necessary for signaling via ErbB4 homodimers and the ability to activate STAT5 but does not affect the ability of ErbB4 to heterodimerize with ErbB2 and to promote signaling via Erk and Akt pathways (Tvorogov et al 2009). Thus, these two kinase-dead somatic mutants of ErbB4 may actually shift the balance of signaling outcome to favor Erk- and Akt-mediated growth and survival, as opposed to STAT5-dependent signaling that, in the context of ErbB4, has been associated with differentiation (Muraoka-Cook et al 2008b, Tvorogov et al 2009).

Three recently described SNPs in the intron sequence of the ErbB4 gene have been shown to associate with breast cancer (Murabito et al 2007). The ErbB4 -782 G>T polymorphism has been identified as a risk allele for breast and colorectal cancer (Rokavec et al 2007). The relevance of these intron sequence allele variants in the regulation of ErbB4 gene trasncription is not known. However, Law et al. have reported that schizophrenia-associated intronic variants in the ErbB4 gene are related to altered ErbB4 splice-variant expression (Law et al 2007).

[1] Oncogene addiction: acquired dependence of tumor cells on an activated oncogene for their survival/proliferation

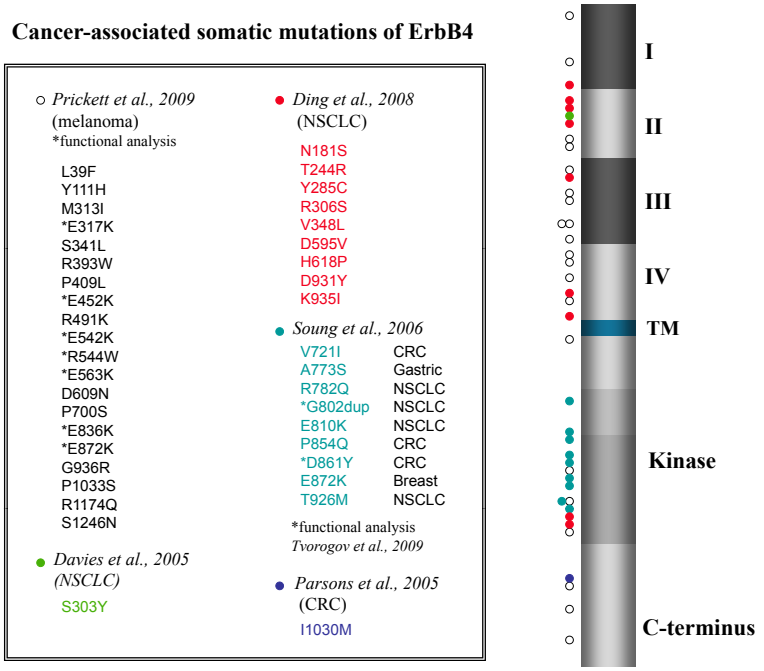

Figure 6 . Cancer-associated somatic mutations of ErbB4
Somatic mutations of ErbB4 observed in melanoma, non-small cell lung cancer (NSCLC), colorectal carcinoma (CRC), breast, and gastric cancer. The mutations are indicated by dots on the ErbB4 structure and located in regions important for ErbB4 activation: domain I or III (ligand-binding), domain II or IV (dimerization), or kinase domain (phosphorylation). TM indicates the transmembrane domain.

2.4.6 Different activities of the ErbB4 isoforms for tumorigenesis

Several reports studying the activities of ligand stimulated ErbB4 demonstrate suppression in breast cancer cell growth. ErbB4 ligands have been reported to delay mitotic progression and induce differentiation or apoptosis (Feng et al 2007, Muraoka-Cook et al 2006a, Muraoka-Cook et al 2006b, Naresh et al 2006, Ni et al 2001, Sartor et al 2001). Also ectopic expression of a constitutively active ErbB4 (JM-a CYT-1) mutant, I658E, promotes tumor cell apoptosis (Vidal et al 2007). However, cre-lox-mediated deletion of ErbB4 has no effect on the occurrence of mammary tumors induced by the *neu* transgene in mice (Jackson-Fisher et al 2006) arguing against a tumor-suppressor role for ErbB4 *in vivo*.

Direct downregulation of ErbB4 expression in cells mostly indicate a tumor growth-supporting role for ErbB4. Inhibition of ErbB4 activity with specific

ribozymes or siRNA decreases proliferation and tumor formation of breast cancer cells both *in vitro* and *in vivo* (Tang et al 1998, Tang et al 1999). RNA intereference approaches also demonstrate that ErbB4 is necessary for survival of Ewing sarcoma cells (Kang et al 2007), as well as of both normal colon epithelial (Frey et al 2009) and colorectal carcinoma (Lee et al 2009) cells. ErbB4-specific siRNAs as well as the ErbB inhibitor lapatinib suppress growth of melanoma cell lines with the activating ErbB4 mutations (Prickett et al 2009). Inhibiting ErbB4 activities with specific antibodies targeting the ligand-binding domain has been shown to block tumor formation of lung and prostate cancer cells *in vitro* and *in vivo* (Starr et al 2006, Vexler et al 2008) by stimulating lung cancer cell apoptosis (Starr et al 2006) and sensitizing prostate cancer cells to radiation therapy (Vexler et al 2008).

Approaches to overexpress wild type ErbB4 in different cellular backgrounds have demonstrated either growth and survival advantage (Alaoui-Jamali et al 2003, Määttä et al 2006, Starr et al 2006, Zhang et al 1996), or enhanced apoptosis (Naresh et al 2006) for cells expressing greater amounts of ErbB4. One explanation for the variable observations may be that different ErbB4 isoforms promote different cellular responses (Muraoka-Cook et al 2009, Määttä et al 2006). For example, overexpression of the cleavable JM-a CYT-2 isoform or the sole CYT-2-type of soluble ICD promotes growth of breast cancer cells *in vitro* (Junttila et al 2005) and of normal mammary epithelium *in vivo* (Muraoka-Cook et al 2009). In contrast, the CYT-1 ICD promotes mammary epithelial cell differentiation (Muraoka-Cook et al 2009) or apoptosis (Naresh et al 2006).

3 AIMS OF THE PRESENT STUDY

The present study aimed to discover new and more specific ways to inhibit ErbB-dependent tumorigenesis and to find out special characteristics of the ErbB4 isoforms and their significance in cancer biology. The specific aims were to:

1. Address the potential of ErbB4 as a target for immunotherapy in cancer

2. Determine the functions of ErbB4 isoforms and cleavage in tumorigenesis

3. Analyze the potential of targeting ErbB2 in gastric cancer

4 MATERIALS AND METHODS

This section is a general view of the materials and methods used. The more specific descriptions of the methods can be found in the original publications (I-III).

4.1 Antibodies (I-III)

4.1.1 Antibodies obtained from collaborators or commercial sources (I-III)

Table 5. Primary antibodies

Antigen	Antibody (application)	Species and source	Used in
ErbB4 (C-terminus)	HFR-1 (IP, IF, IHC)	mouse mAb, Neomarkers	I, II
ErbB4 (C-terminus)	E-200 (W)	rabbit mAb, Abcam	I, II
ErbB4 (C-terminus)	Sc-283 (W, cELISA)	rabbit pAb, Santa Cruz	I, III
p-ErbB4 (C-terminus)	pTyr1284 (W)	mouse mAb, Cell Signaling	I
EGFR	Sc-03 (W, cELISA)	rabbit pAb, Santa Cruz	I, III
ErbB2	Sc-284 (W, cELISA)	rabbit pAb, Santa Cruz	I, III
ErbB2 (extracellular)	2C4 (MTS)	mouse mAb, Genentech	I
ErbB2 (extracellular)	Trastuzumab (MTS, Xn)	zumab, Roche	III
ErbB3	Sc-285 (W, cELISA)	rabbit pAb, Santa Cruz	I, III
Actin	Sc-1616 (W)	goat pAb, Santa Cruz	I, II
CD44	Hermes-3 (IHC)	mouse mAb, Dr. S. Jalkanen	I, II
T cell receptor (chicken)	3g6 (IgG control)	mouse mAb, Dr. S. Jalkanen	I, II
No reactivity against human/mouse tissues	AK990/02 (IgG control) (Xn)	mouse mAb, Invivo Biotech Services	II
His	Anti-penta His (W)	mouse mAb, Qiagen	I
Flag	Anti-Flag (W)	mouse mAb, Sigma	I
CD20	Rituximab (Xn)	ximab, Roche	III
Hemagglutinin	Anti-HA (IF)	rat mAb, Roche	I
Digoxigenin	Anti-digoxigenin	mouse mAb, Roche	III

mAb monoclonal antibody, *pAb* polyclonal antibody, *zumab* humanized monoclonal antibody, *ximab* chimerized monoclonal antibody, *W* Western blot analysis, *IP* immunoprecipitation, *IF* immunofluorescence, *IHC* immunohistochemistry, *Xn* xenografts, *MTS* proliferation assay, *cELISA* cell enzyme-linked immunosorbent assay

4.1.2 Generation of mAbs against the extracellular domain of ErbB4 (I)

Specific oligonucleotides were synthesized on the basis of the ErbB4 cDNA sequence (Plowman et al 1993). Total cellular RNA was extracted from MDA-MB-453 cells and used as a template in RT-PCR, to generate the human ErbB4 ECD coding sequence. Antigen purification was accomplished utilizing anti-gD affinity chromatography (Paborsky et al 1990). For the generation of ErbB4 monoclonal antibodies Balb/c mice were immunized with approximately 5 μg of ErbB4 ECD in RIBI MPL+TDM+ CWS Emulsion (RIBI ImmunoChem Research Inc., Hamilton, MT) in their rear footpads on weeks 0,1,2 and 3. The immunized mice were tested for an antibody response by ELISA assay. The mice with the highest titers were given an additional 5 μg of ErbB4 ECD in RIBI during week 4. Three days later, the lymphocytes from the popliteal and inguinal nodes, were fused with mouse myeloma line X63-Ag8.653 (Kearney et al 1979), using 50% polyethylene glycol 4000 (Boehringer Mannheim Corporation, Indianapolis, IN) as previously described (Oi et al 1980). Fused cells were plated at a density of 200,000 cells per well in 96-well tissue culture plates and hybridoma selection using HAT media supplement (Sigma, St. Louis, MO) was initiated one day after fusion. Beginning on day 10, the hybridoma supernatants were screened for the presence of ErbB4-specific antibodies using a radioactive capture assay as described below. Stable antibody producing clones were obtained by limiting dilution and large quantites of specific mAbs were produced in ascites. The antibodies were purified on protein A-Sepharose columns (Fermentech, Inc., Edinburgh, UK) and stored in PBS at 4°C.

For the radioactive capture assay, maxisorp breakapart modules (Nunc, Roskilde, Denmark) were coated with 100 μl of 2 μg/ml goat anti-mouse IgG (Boehringer, Mannheim, Germany) overnight at 4°C. The plates were washed with PBS/0.5% Tween 20 (TBST), blocked with ELISA diluent (PBS/0.5%BSA/0.05% Tween 20) and incubated with monoclonal supernatants for 2 hr at room temperature. The plates were washed and incubated for an additional hour with 40,000 counts/ well of [^{125}I]ErbB4 ECD, radiolabelled by lactoperoxidase. After washing, the amount of ErbB4 bound to the antibodies was determined by counting the wells on a Wallac 1277 GammaMaster (Wallac Inc, Gaithersburg, MD).

Twenty-nine hybridoma clones secreting antibodies were obtained of which five were further characterized and used in the original publications I and II. Information about the other specific anti-ErbB4 mAbs are listed in the supplementary data of original publication I.

Table 6. Anti-ErbB4 monoclonals (I, II)

Name	Isotype	Epitope	Application (used in)
mAb 1479	IgG2a	Domain IV, D	Characterization, functional assays *in vitro* (I) and *in vivo* (II)
mAb 1475	IgG2b	P	Effect on ErbB4 cleavage (I)
mAb 1464	IgG2b	C	Western analysis, non-reduced (I, II)
mAb 1536	IgG2b	A	Serum ELISA (II)
mAb 1482	IgG2b	H	Serum ELISA (II)

Isotyping of the monoclonal antibodies was done using Mouse MonoAb ID/SP isotyping kit (Zymed, South San Francisco, CA) according to the manufacturer's instructions. The epitope mapping was performed by cross-blocking ELISA. The ErbB4 mAbs were grouped into epitopes based on their ability to block binding of the others by 50% or more in comparison to an irrelevant mAb control.

4.1.3 Secondary antibodies (I-III)

HRP conjugated antibodies against mouse, rabbit and goat were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and the anti-mouse peroxidase polymer was from Immunovision Inc. (Daly City, CA).

Biotinylated anti-mouse IgGs were from Vector Laboratories (Burlingame, CA).

Fluorescence-labeled Alexa Fluor goat 488 anti-mouse and Alexa Fluor 568 goat anti-rat were both from Molecular Probes (Leiden, the Netherlands).

4.2 Reagents and chemicals (I, II)

Table 7. Reagents and chemicals

Compound	Application	Supplier	Used in
NRG-1 (50 ng/ml)	Stimulation of ErbB4 phosphorylation	R&D	I
PMA (TPA) (100 ng/ml)	Stimulation of ErbB4 cleavage	Sigma	I
G418 (200 ng/ml)	Selection of transfected cells	Calbiochem	I
Biotin-NHS (1 mg/ml in DMSO)	Biotinylation of mAb 1482	Calbiochem	II

R&D Minneapolis, MN *Sigma-Aldrich* St Louis, MO *Calbiochem* Darmstadt, Germany

4.3 Clinical samples (I-III)

Use of all tissue samples was approved by Institutional Review Boards or Institutional Ethics Committees. All study participants provided written informed consent before entry.

I Frozen sections of normal human heart and kidney were obtained from a two year old male died of an electric shock. Seventeen snap-frozen tissue sample pairs representing human breast cancer and histologically normal peripheral tissue from the same patient were kindly provided by Dr. Manolo M. Morente (Spanish National Tumour Bank Network, Spanish National Cancer Centre (CNIO), Madrid, Spain).

II Serum samples and the corresponding paraffin sections of 243 breast cancer patients who had undergone surgery in 2004 were collected in University Hospital Leuven, Belgium (Brouckaert et al 2009). In addition, 20 serum samples representing primary and 10 samples representing metastatic breast cancer were obtained from University Hospital of Helsinki, Finland (Joensuu et al 2009). As a control, 30 serum samples were collected from healthy volunteers (24 females, 6 males) in Turku, Finland.

III One-hundred and thirty-one gastric adenocarcinoma patients who had undergone surgery between 1981 and 1995 were identified from the specimen archive of the Department of Pathology, Tampere University Hospital, Tampere, Finland. One-hundred patients with esophagogastric adenocarcinoma with the primary tumor located either in the cardia (55 cases) or in the distal third of the esophagus (45 cases) who had undergone diagnostic endoscopy or surgery between 1990 and 1998 were identified from the specimen archive of the Department of Pathology, Helsinki University Hospital, Helsinki, Finland.

4.4 Cell culture (I-III)

Table 8. Cell lines

Cell line	Species	Cell type	Culture conditions	Used in
T-47D	human	breast cancer	RPMI, 10% FCS, 1% GPS	I-III
MCF-7	human	breast cancer	RPMI, 10% FCS, 1% GPS, 1 nM estrogen, 10 nM insulin	I
COS-7	monkey	kidney fibroblast-like	DMEM, 10% FCS, 1% GPS	I, II
NIH 3T3-7d	mouse	fibroblasts	DMEM, 10% FCS, 1% GPS	I
HEK-293 EBNA	human	embryonal kidney (Invitrogen)	DMEM, 5% FCS, 1% GPS, 200 μg/ml G418	I
NR6	mouse	fibroblasts	DMEM, 10% FCS, 1% GPS	I
N87	human	gastric cancer	RPMI, 10% FCS, 1% GPS	III
SKBR-3	human	breast cancer	RPMI, 10% FCS, 1% GPS	III

FCS fetal calf serum, *GPS* (200 mM L-glutamine, 100 IU/ml penicillin, 100 mg/ml streptomycin)

4.5 Plasmid constructs and transfection (I, II)

Full-length ErbB4

pcDNA3.1ErbB4JM-aCYT-2, pcDNA3.1ErbB4JM-bCYT-2 (Määttä et al 2006)

pcDNA3.1ErbB4JM-aCYT-2-HA, pcDNA3.1ErbB4JM-bCYT-2-HA; hemagglutinin tagged (Sundvall et al 2007)

pcDNA3.1ErbB4JM-aCYT-2-K751R; kinase dead, produced using site-directed mutagenesis kit (Stratagene, La Jolla, CA)

HIS-tagged ErbB4 ectodomain

pcDNA3.1ErbB4ECD-HIS, derived from full length *pcDNA3.1ErbB4JM-aCYT-1* using:

5'-primer TTGGTACCGCACCATGAAGCCGGCGACAGGAC

3'-primer TTATCTCGAGTTAGTGATGGTGATGGTGATGTTGTGGTAA AGTGGAATG

HIS-tagged ErbB4 extracellular domain (I-IV) constructs

pcDNA3.1ErbB4ECDI-HIS, derived from full length *pcDNA3.1ErbB4JM-aCYT-1* using:

5'-primer GAGGCGGCCGCCTGTGCAGGAACGGAGAATAAAC

3'-primer CTACTTAAGTTAATGGTGATGGTGATGGTGTCCTGAACTAC CATTTGTTGAC

pcDNA3.1ErbB4ECDII-HIS

5'-primer GAGGCGGCCGCCTGTGGACGTTGCCATAAGTCC
3'-primer CTACTTAAGTTAATGGTGATGGTGATGGTGACAAGCTTTTG GGCAAATGTCAG

pcDNA3.1ErbB4ECDIII-HIS

5'-primer GAGGCGGCCGCCGATGGCATTGGCACAGGATC
3'-primer CTACTTAAGTTAATGGTGATGGTGATGGTGATTTTCAGCTTT TCTGTTGTCCCG

pcDNA3.1ErbB4ECDIV-HIS

5'-primer GAGGCGGCCGCCTGTACTGCTGAAGGAATGGTG
3'-primer CTACTTAAGTTAATGGTGATGGTGATGGTGTCTAGCATGTT GTGGTAAAGTGG

Cells plated on 24-well plates (4 x 10^4) or 6-well plates (1.5 x 10^5) were transfected with 0.5-1 μg of appropriate plasmids using FuGENE 6 trasnfection reagent (Roche, Mannheim, Germany).

4.6 Production of recombinant extracellular domain of ErbB4 (I)

HEK293 EBNA cells transfected with pcDNA3.1*ErbB4ECD-HIS* were selected in medium containing 150 μg/ml Hygromycin B (Roche) and after cloning maintained in the presence 75 μg/ml of Hygromycin B. The HIS-tagged ectodomain was purified from culture medium containing 0.5% FCS by immobilized metal chelate affinity chromatography (GE Healthcare, Chalfont St Giles, UK) by stepwise pH gradient.

4.7 Real-time RT-PCR (III)

The design and evaluation of specificity of the oligonucleotide probes used to quantitate ErbB receptor mRNA levels is described in detail elsewhere (Junttila et al 2003). Total RNA was extracted from cell cultures using RNAzol B reagent (Tel-Test Inc., Gainesville, FL) according to the manufacturer's instructions. After treatment with DNAse 1 (Life Technologies Inc., Rockville, MD), 1 μg of total RNA was subjected to cDNA synthesis using random hexamer primers (Promega, Madison, WI) and M-MLV RNase H minus reverse transcriptase (Promega) in a total volume of 25 μl according to the manufacturer's protocol. Taqman quantitative RT-PCR analysis was carried out using 300 nM of primers, 200 nM of probes, 12.5 μl of Taqman universal PCR Master Mix (PE Biosystems, Foster City, CA) and 0.5 μl of reverse transcribed template cDNA in a total volume of 25 μl. Thermal cycling (ABI PRISM 7700 Sequence Detector; PE Biosystems) was started with initial steps of 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60°C. C_T values (the cycle number at which the signal exceeds a fixed threshold

value) were determined and normalized against an internal control (β-actin mRNA expression) from parallel samples.

4.8 Immunoprecipitation and Western blot analyses (I-III)

Cell and tissue samples were lysed in lysis buffer and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting as previously described (Kainulainen et al 2000). Whenever the monoclonal ErbB4 ectodomain antibodies were used as the primary antibodies in Western blotting dithiothreitol (DTT) was excluded from the sample loading buffer and samples were not subjected to boiling. Protein immunoprecipitations were accomplished with G-sepharose beeds (GE Healthcare) and specific antibodies.

4.9 Immunohistochemistry and CISH (I-III)

Frozen and paraffin embedded sections of 5 μm were stained according to standard procedures using ABC kit (avidin-biotin complex; Vector Laboratories) to detect the biotinylated secondary antibody and diaminobenzidine peroxidase substrate (DAB; Vector Laboratories) as a chromogen.

For chromogenic in situ hybridization (CISH), sections were heated at 94 °C for 3 min, and hybridized with digoxigenin-labeled ErbB2 or Topoisomerase IIα DNA overnight at 37 °C. The probes were detected by sequential incubations with mouse anti-digoxigenin, anti-mouse-peroxidase polymer and DAB.

4.10 Immunofluorescence staining (I)

For immunofluorescence staining, cells were fixed and permeabilized with methanol for 15 minutes at -20°C. After washing the cells twice with PBS, a dilution of the primary antibody was directly applied on the coverslips/slides and incubated for 1 hour in PBS containing 1% BSA following a 30 minute incubation with a fluorescent labeled secondary antibody. The coverslips were mounted with Vectashield mounting medium (Vector Laboratories).

4.11 Proliferation and anchorage-independent growth assays (I, III)

Cell proliferation was estimated with CellTiter 96 Non-Radioactive Cell Proliferation Assay (Promega) according to the manufacturer's instructions. Cells were starved overnight and plated at a density of 1-1.5x10^4 on 96 well plates in culture medium containing 5% charcoal-stripped fetal calf serum and the monoclonal antibodies described in original publications I and III. Cell number was also estimated using a hemocytometer from six-well plates plated at a density of 1x10^5 cells/well.

For the anchorage independent growth assays bottom layers consisting of 2 ml RPMI, 0.5% Bacto agar, and 10% FCS were applied on six-well plates. After solidification of the bottom layers, top layers consisting of $3x10^4$ cells/well in 1.2 ml RPMI, 0.33% Bacto Agar, and 10% FCS with or without 1 μg/ml mAb 1479 or the control antibody 3g6, were applied. Cells were incubated for 14 days at 37 °C. Colonies larger than eight cells were counted under microscope.

4.12 ELISA (I, II)

4.12.1 Cell and recombinant ErbB4 coated ELISAs (I)

NIH 3T3 and NR6 cells were plated (3×10^4 cells/well) on white flat bottom 96-well plates (Perkin Elmer, Zaventem, Belgium). Cells were fixed and permeabilized with methanol and stained with primary antibodies followed by incubation with HRP-conjugated secondary antibodies. Luminescence signal was obtained using a chemiluminescence substrate (Thermo Scientific, Rockford, IL) and measured by TECAN Ultra (Tecan Nordic AB, Mölndal, Sweden).

To determine the affinity of mAb 1479 for the ErbB4 ectodomain, 100 ng of the HIS-tagged recombinant ectodomain was coated on white flat bottom 96-well plates in a buffer containing 100 mM $NaHCO_3$ (pH 9.6). The plates were washed with washing solution (0.1% Tween in PBS), and blocked with blocking solution (5% milk and 1% BSA in TBST (0.88% NaCl, 10 mM Tris-HCL, pH 7.5, 0.05% Tween 20)) following incubation with different concentrations of mAb 1479 or a negative control antibody (3g6; 100 nM) diluted in blocking solution. Luminescence was measured as above and the background signal of 3g6 was subtracted before a non-linear curve analysis with OriginPro7 (OriginLab Corporation, Northampton, MA).

4.12.2 Sandwich ELISA (II)

For serum ELISA, mAb 1482 (diluted 1 mg/ml in PBS pH 8.5) was biotinylated with 18% Biotin-NHS reagent and incubated on a shaker for four hours at room temperature. The unbound biotin was removed by dialysis.

Wells of white flat bottom 96-well plates were coated with 100 μl of mAb 1536 or the negative control 3g6 at 10 μg/ml in 0.1 M $NaHCO_3$ (pH 9.6) overnight at 4 °C and then at 37 °C for 1 hour. The wells were washed six times with washing solution (0.1% Tween 20 in PBS) and then blocked to prevent non-specific absorption by the addition of 200 μl of blocking solution (PBS containing 1% gelatin and 1% nonfat milk) for 45 minutes at room temperature. After washing the wells four times with washing solution, 100 μl of each serum sample (at 1:25 dilution in washing solution) was added into the wells and incubated for 1 hour at room temperature. The wells were washed again four

times and incubated with 100 µl of the biotinylated mAb 1482 (10 µg/ml in washing solution) at room temperature for 1 hour. After four washes, 100 µl of strepavidin-horseradish peroxidase diluted 1:1000 in washing solution was added and the plates were incubated for 1 hour at room temperature. The plates were washed six times and finally developed with ELISA POD chemiluminescence reagent according to the manufacturer's instructions (Roche). The luminescence signals of the solutions were measured after a 3-minute incubation series with TECAN Ultra.

Each assay included a titration series of the recombinant ErbB4 extracellular domain that was used to generate a standard curve. To obtain a protein milieu similar to the serum samples the titration standards were diluted in washing solution containing human AB serum (PromoCell GmbH, Heidelberg, Germany) that did not contain detectable levels of ErbB4 ectodomain (data not shown).

4.13 Animal experiments (II, III)

All animal experiments were approved by an Ethics Committee.

4.13.1 Breast cancer xenografts treated with mAb 1479 (II)

A human breast cancer cell line (T-47D) expressing endogenous ErbB4 was used to test the *in vivo* effects of mAb 1479 in inhibiting tumor growth. Two million cells in a total volume of 50 µl (50% culture medium and 50% matrigel (BD Biosciences, San Jose, CA)) were inoculated into the mammary fat pads of six-week old female SCID mice (Charles River, Sulzfeld, Germany). The following day, the mice were treated intraperitoneally either with 5 mg/kg of mAb 1479 or a control antibody AK990/02. The treatment was given every fourth day and continued for three weeks. The mice were held an additional three weeks untreated and then sacrificed. The tumors were measured by a digital caliper and tumor volume was calculated using the formula $T_{vol}=\pi/6$ x larger diameter x (smaller diameter)^2. Thereafter, the tumors were lysed in lysis buffer and subjected to Western blot analysis for ErbB4 expression.

4.13.2 Gastric cancer xenografts treated with trastuzumab (III)

N87 cell were tested for trastuzumab sensitivity when grown as xenograft tumors in female nu/nmri nude mice (Harlan Netherlands, Horst, The Netherlands). Trastuzumab treatment was initiated 14 days post-inoculation and given intraperitoneally at a weekly dose of 5 mg/kg for five weeks until sacrificed. The effects of tratsuzumab were compared with the same dose of a similar humanized antibody rituximab, which is directed against CD20 differentation antigen not expressed in carcinoma cells. Tumor growth was measured using a caliper and tumor volume was calculated as above.

5 RESULTS

5.1 Specific inhibition of the ErbB4 JM-a isoform suppresses breast cancer cell growth (I, II)

Several issues can reason the development of therapeutic tools that specifically target ErbB4 JM-a isoform in cancer. This isoform is commonly overexpressed in different cancers (Junttila et al 2005). Its isoform specific activities and nuclear signaling are associated with growth promoting effects in cancer cells (Määttä et al 2006), and nuclear immunoreactivity of ErbB4 is associated with less favorable prognosis in breast cancer compared to ErbB4 at the cell surface (Junttila et al 2005). Moreover, the ErbB4 isoforms have different tissue distribution. Thus, targeting of the JM-a isoform may be directed more efficiently to diseased tissues with less severe side effects from other tissues.

Human recombinant ErbB4 extracellular domain of JM-a isoform was used to immunize mice for the generation of hybridoma clones secreting monoclonal antibodies that specifically recognize the JM-a isoform of ErbB4. For the initial screening of mAbs by Western blot, COS-7 cells were transfected either with the full-length ErbB4 JM-a or JM-b extracellular variants or a vector control. One of the antibodies, mAb 1479, solely recognized the JM-a transfected cells when used as the primary antibody in Western blotting (I: Fig 1A). The signal obtained with mAb 1479 was reduced in intensity when the protein samples were subjected to reducing conditions. Thus, the interaction of mAb 1479 with ErbB4 extracellular domain is highly conformation-specific. The structurally native full-length ErbB4 protein in non-reducing conditions appeared as a band of 150 kDa compared to the normally observed band of 180 kDa in Western blotting under reducing conditions (I: Fig 1A, lane 7 and 8). The specificity of mAb 1479 for JM-a isoform was confirmed by immunofluorescence staining of the same cells transfected with hemagglutinin (HA)-tagged full-length isoform variants (I: Fig 1B). Again, specific staining with mAb 1479 was demonstrated in the JM-a transfectants that stained positive also for the HA-tag. A commercially available ErbB4 antibody (HFR-1) against the ErbB4 intracellular domain recognized both JM-a and JM-b transfected cells.

To exclude any cross-reactivity of mAb 1479 with other ErbB receptors, NIH 3T3-7d and NR6 transfectants expressing the different ErbB receptors were used for Western blotting and cell-based ELISA. In the Western analysis mAb 1479 gave a signal solely for cells expressing ErbB4 except for a weak band observed in cells expressing EGFR (I: Fig 1C). To exclude the amount of cross-reactivity of mAb 1479 with EGFR, a cell-based ELISA with the same cells was performed with three different consentrations (1:1000, 1:500 and 1:100) of each ErbB antibody (I: Fig 1D). Even in the highest concentration (1:100) mAb 1479 gave a signal only for NR6 cells expressing the ErbB4 JM-a isoform.

To more precisely characterize the interaction of mAb 1479 with the extracellular domain of ErbB4, a recombinant His-tagged ErbB4 extracellular domain was incubated in the presence of mAb 1479 *in vitro*. The formed complex of 250 kDa was visualized by Western blotting under non-reducing conditions (I: Fig 2C). To determine the affinity of mAb 1479 binding to the extracellular domain of ErbB4, ErbB4 ectodomain was immobilized onto microwells and incubated in the presence of mAb 1479 concentration ranging from 0.195 to 100 nM. mAb 1479 bound ErbB4 with a K_d value of 0.85 nM ± 0.077 (I: Fig 2D). The epitope of mAb 1479 on the extracellular domain of ErbB4 was mapped using four different His-tagged constructs comprising of the different extracellular domains I-IV (II: Fig 3A). mAb 1479 only recognized the domain IV in Western analysis (II: Fig 3B).

Encouraged by these *in vitro* results of mAb 1479 specificity for the JM-a isoform, the *in vivo* specificity was analyzed. Based on the earlier observation that kidney expresses only the JM-a isoform and heart tissue solely the JM-b isoform of ErbB4 (Junttila et al 2005), frozen sections of these human tissues were stained with mAb 1479 as the primary antibody. mAb 1479 signal was only present in kidney tissue as compared to staining of both tissues using a commercial ErbB4 antibody recognizing an intracellular epitope on ErbB4 (HFR-1) (I: Fig 2A). Interestingly, these two antibodies showed different ErbB4 staining patterns. This might result from the different epitopes they recognize but also from the fact that kidney tissue expresses the cleavable isoform that can be shed as a soluble extracellular domain into the surrounding extracellular space. When the kidney tissue lysate was studied for ErbB4 expression and cleavage by Western analysis with mAb 1479 as the primary antibody (I: Fig 2B) the shed ectodomain was seen to comprise a high proportion of the ErbB4 content in kidney tissue.

As mAb 1479 targets the extracellular domain IV of ErbB4 its functions on ErbB4 activity and cleavage were analyzed. MCF-7 breast cancer cells expressing endogenously the cleavable JM-a isoform were treated with different concentrations of mAb 1479 before stimulating ErbB4 phosphorylation with its ligand NRG-1. mAb 1479 was effective in suppressing ErbB4 phosphorylation in a time-dependent manner (I: Fig 4A). Because ErbB4 activation has been demonstrated to increase ErbB4 cleavage, the effect of mAb 1479 on both basal and PMA-stimulated ErbB4 shedding was tested. COS-7 cells transfected with the full-length JM-a CYT-2 isoform were treated with mAb 1479 for 24 hours prior to Western blot analysis of ErbB4 shedding and cleavage. The shed ErbB4 ectodomain was detected from the medium of untreated or mAb 1475 (control) treated COS-7 cells but hardly any signal of the ectodomain was observed in mAb 1479 treated cells (I: Fig 4B), indicating a suppressive effect for mAb 1479 on ErbB4 shedding. No significant effect was seen on the levels of cell-associated 180 or 80 kDa species under these conditions. In this experiment,

mAb 1479 was actually observed to become rapidly depleted from the culture medium of cells expressing ErbB4 but not from vector transfected cells (data not shown). In fact, it was efficiently internalized in an ErbB4-dependent manner (I: Fig 5A). The internalized antibody was found to co-localize with Rab5-positive early endosomal vesicles and the internalization of mAb 1479 via ErbB4 was not dependent on ErbB4 tyrosine kinase activity as cells transfected with a kinase dead ErbB4 construct were as effective in internalizing mAb 1479 as was the wild type ErbB4 construct (I: Fig 5A). Furthermore, mAb 1479 treatment increased ErbB4 ubiquitination of both cytosolic isoforms (I: Fig 5B) and significantly decreased steady state levels of ErbB4 protein (I: Fig 5C). The effect on ErbB4 downregulation was already seen 2 h after adding mAb 1479 in MCF-7 cell culture media and lasted for 72 hours.

To determine the significance of inhibiting ErbB4 activities for breast cancer cell growth we studied the effects of mAb 1479 on two breast cancer cell lines in proliferation and soft agar assays *in vitro*. Both cell lines demonstrated sensitivity to mAb 1479. However the effect in suppressing breast cancer cell proliferation (I: Fig 6A) and anchorage-independent growth (I: Fig 6B and C) was more significant in T-47D cells than in MCF-7 cells. Because the T-47D cell line was more sensitive to the effects of mAb 1479 this cell line was used to study the *in vivo* effects of mAb 1479 in a mouse xenograft model of mammary tumor formation. A three week period of mAb 1479 treatment significantly decreased tumor volume (P = 0.047) compared to the control IgG treated animals. The control tumors grew progressively and reached a mean volume of 17 mm^3 (range 3-47 mm^3; n = 7) in 42 days. The mAb 1479-treated xenografts only reached a mean volume of 3.1 mm^3 (range 1.4-5.6 mm^3; n = 5) (II: Fig 1 A).

The xenograft tumors were also subjected to ErbB4 protein analysis by Western blotting to observe the effects of mAb 1479 on ErbB4 *in vivo*. The Western analysis showed that the major ErbB4 species in these xenograft tumors was actually the 80 kDa ErbB4 fragment. This was different from the ErbB4 species in adherent T-47D cells *in vitro* that demonstrate hardly any detectable 80 kDa ErbB4 (II: Supplemental Fig 1). Treatment of the xenografts with mAb 1479 reduced the relative proportion of the 80 kDa fragment of total ErbB4 from an average of 90% to 74% (P = 0.01) (II: Fig 2A and B). As other factors in addition to ErbB4 cleavage by TACE can regulate the accumulation of the 80 kDa fragment, the amount of shed ErbB4 ectodomain was also assessed by Western analysis. Again, mAb 1479 significantly reduced the relative proportion of the ectodomain fragment of total ErbB4 from 38% to 9% (P = 0.01) (II: Fig 2C and D). These data indicate efficient inhibition of ErbB4 activities by mAb 1479 both *in vitro* and *in vivo* and are consistent with a role of ErbB4 proteolytic processing for tumor growth.

5.2 ErbB4 shedding is increased in breast cancer (I-II)

The tumor-promoting activities of the JM-a isoform have been described to include cleavage and the translocation of the nuclear intracellular receptor fragment (Määttä et al 2006). Tumors that manifest ErbB4 cleavage may serve as potential targets for mAb 1479. Thus, there was a rationale to first determine the frequency, extent and clinical associations of ErbB4 cleavage in breast cancer and secondly identify patients demonstrating cleavage that could possibly benefit of JM-a targeted therapy. To untangle these questions 17 snap-frozen tissue sample pairs representing human breast cancer and histologically normal peripheral tissue from the same patient were analyzed for ErbB4 shedding by Western blot analysis with mAb 1479 (I: Fig 3A). The signal for ErbB4 ectodomain was observed in 75% of the tumor samples and only 18% of the normal tissues. By densitometric quantification the ectodomain signal ratio against total ErbB4 signal in tumor compared to normal tissue was significantly increased (P = 0.015) (I: Fig 3C). This demonstrates that transformation of histologically normal tissue to breast carcinoma is associated with enhanced shedding.

The observed increase of ErbB4 shedding in breast cancer may be causally linked to the nuclear accumulation of ErbB4, a phenomenon previously described in a subpopulation of ErbB4-positive breast cancers with relatively less favorable prognosis (Junttila et al 2005). To test this causality of increased ErbB4 shedding and nuclear accumulation an ELISA-based technique was developed to measure the amount of shed soluble ectodomain from breast cancer patient serum samples utilizing the previously screened monoclonal antibodies against different epitopes on the ErbB4 ectodomain (I: Supplemental table 1). The cut-off level for ErbB4ELISA to separate cancer serum ErbB4 levels from normal ErbB4 levels was determined by ROC curve analysis (II: Fig 5). In both groups some samples were not measurable or did not give a signal (under the detection limit of the assay 1 ng/ml), and were excluded from the analysis. The area under curve (AUC) in ROC was 0.76 indicating a reasonable resolution power for the different ErbB4 ectodomain levels (II: Fig 5). The best combination in sensitivity and specificity was obtained at 40 ng/ml.

The analysis of 238 breast cancer patient serum samples by ELISA from a total series of 243 breast cancer patients demonstrated ErbB4 ectodomain levels to be significantly higher in 50 patients (21%, cut-off >40 ng/ml) of the cancer serum samples compared to non of the 30 normal healthy controls (P = 0.0021) (II: Fig 4A). The median of normal serum ectodomain level was 9.9 ng/ml (+/- 11.6 ng/ml).

In parallel to ELISA, the corresponding tumor tissues of the series of 243 patients were stained for ErbB4 by immunohistochemistry. The observed ErbB4

signal was scored based on intensity of both cytoplasmic and nuclear staining as either negative (0), weak (1), moderate (2) and strong (3). Samples that stained negative contained ≤10% ErbB4-positive tumor cells. Strong cytoplasmic ErbB4 signal was detected in 44 (18%) of the samples, moderate in 115 (48%), weak in 60 (25%), and totally negative were 21/243 (8.6%) samples. Strong nuclear ErbB4 expression was present in 21 (9%), moderate in 37 (15%), weak in 67 (28%) cases, and no nuclear ErbB4 expression was detected in 115 (48%) tumors (II: Fig 6). All ErbB4-positive tumors showed cytosolic immunostaining, whereas none of the tumors had immunoreactivity solely in the nucleus.

The immunohistochemically detected total ErbB4 expression associated with ER-positivity ($P = 0.001$) and a high histological grade of differentiation ($P = 0.004$). The nuclear signal alone did not, however, associate with ER-positivity ($P = 0.092$) or grade ($P = 0.065$) and might indicate different prognostic significance for ErbB4 staining in the nuclear compartment. The different levels of ErbB4 ectodomain (high ≥40 ng/ml and low <40 ng/ml) had no significant associations with the clinical parameters tested. Also, no significant association between high levels of ErbB4 ectodomain and total ErbB4 IHC expression ($P = 0.50$) or nuclear accumulation ($P = 0.94$) was seen. However, there was a trend between lower levels of ErbB4 ectodomain in the postmenopausal patients as compared with premenopausal patients ($P = 0.06$). The relatively short follow-up (median five years) of the present series precluded from analysing the effects of different ErbB4 expression levels on disease-free survival and overall survival as only five patients had died and three relapsed during this period.

This data suggests that ErbB4 ectodomain shedding cannot be used as a biomarker for ErbB4 nuclear accumulation and is consistent with the reported observation that other factors, in addition to proteolytic processing, regulate nuclear accumulation of ErbB4. However, both assays, IHC and ELISA, measure different characteristics of ErbB4 activity in tissues and could have value in patient selection or monitoring treatment efficacy of ErbB4-targeted therapies.

5.3 ErbB2 is amplified in the intestinal type gastric cancers (III)

To study the possible role of ErbB2 as a biomarker and target for immunotherapy in gastric cancer ErbB2 and Topoisomerase IIα gene amplification was analyzed from a series of 131 gastric adenocarcinomas and 100 gastroesophageal/cardia cancers by CISH method (III, Supplemental data). ErbB2 amplification was detected in sixteen gastric adenocarcinoma samples (12.2%) and 24 (24%) gastroesophageal cancers. ErbB2 amplification was significantly associated with the intestinal histologic type gastric adenocarcinomas in contrast to the diffuse and mixed /anaplastic types ($P =$

0.0051, III: Table 1). In the same series Topoisomerase IIα gene was co-amplified with ErbB2 in 63% of gastric adenocarcinomas and 67% of gastroesophageal tumors. None of the gastric adenocarcinoma samples without ErbB2 amplification had Topoisomerase IIα gene amplification. The Topoisomerase IIα gene amplification analysis in gastroesophageal cancers was restricted only to the tumors with ErbB2 amplification.

ErbB2 amplification in the gastric adenocarcinomas associated with poor carcinoma-specific survival (P = 0.0089, III: Table 1) and this association was even stronger in the intestinal histologic type of gastric adenocarcinoma (P = 0.0019, III: Fig 1B). However, when the significance of ErbB2 amplification for disease outcome was analyzed the median survival of patients with ErbB2-negative cancer (12.7 months) was almost twice the survival of patients with ErbB2 amplification (6.6 months). This difference was not statistically significant (P = 0.37) and proposes more relevance for ErbB2 as a predictive marker for trastuzumab sensitivity than as a prognostic marker.

5.4 Trastuzumab inhibits growth of a gastric cancer cell line (III)

Based on the observed prevalence of ErbB2 amplification in the studied gastric cancers samples, sensitivity of a gastric cancer cell line (N87) was tested for trastuzumab both *in vitro* and *in vivo*. The expression levels of ErbB2 in N87 cells was first studied and compared with two breast cancer cell lines, one with ErbB2 amplification (SKBR-3) and one without ErbB2 amplification (T-47D). ErbB2 mRNA and protein levels were highly overexpressed in N87 and SKBR-3 compared with T-47D cells (III: Fig 2A and Fig 2C). By using FISH, ErbB2 overexpression in both N87 and SKBR-3 cells was verified to be a result of high level of ErbB2 gene amplification (data not shown). *In vitro* dose-response curves to trastuzumab were determined using a non-radioactive cell titer assay (MTT) and by direct counting of cells with a hemocytometer. These experiments indicated that trastuzumab inhibited growth of N87 and SKBR-3 cells at equal efficacy (III: Fig 3A, data not shown). Trastuzumab was also efficient in inhibiting tumor growth of N87 xenograft tumors when administered as a weekly dose of 5 mg/kg (III: Fig 3B).

6 DISCUSSION

6.1 Antibody-based targeting of ErbB4

The hypothesis of targeting ErbB4 in breast cancer is more complex than targeting other ErbB family receptors as ErbB4 has a relatively controversial role in mammary tumorigenesis (Gullick 2003). Association of ErbB4 with normal mammary gland differentiation (Jones et al 1999, Muraoka-Cook et al 2006b) as well as with ER signaling in breast cancer (Muraoka-Cook et al 2008a, Sundvall et al 2008a) supports a view of ErbB4 as a marker of a well-differentiated phenotype. In fact, the succesful current therapy using anti-estrogens for ER-positive breast cancer may decrease the need in developing additional therapy for this subgroup of patients. Furthermore, ErbB4 ICD has been found to be an important mediator of tamoxifene induced apoptosis and loss of ErbB4 expression in breast cancer serves as a potential biomarker for tamoxifene resistance (Frogne et al 2009, Guler et al 2007, Naresh et al 2008). However, treatment with mAb 1479 demonstrated efficient inhibition of breast tumor growth both *in vitro* an *in vivo*. Thus, it is plausible that some ER-positive tumors actually benefit from targeting ErbB4. In fact, although related to favorable overall survival, some ER-positive cancers relapse despite the available treatment options. It is still not known what factors promote the heterogeneity in the subgroup of well-differentiated tumors. However, nuclear accumulation of ErbB4 has been shown to significantly alter the favorable survival within the ErbB4 positive cancers (Junttila et al 2005). It is also worth investigating the effects of mAb 1479 in combination with tamoxifene to understand the significance of ErbB4 in tamoxifene response. The clinical benefit of targeting only the tumor-associated isoform of ErbB4 is that the treatment can be more specifically localized to diseased tissues reducing the possibility of severe side effects occurring from other tissues.

Monoclonal antibodies targeting ErbB receptors (trastuzumab, cetuximab and panitumumab) have already been introduced into the clinical practice, and have demonstrated both efficacy as well as a favorable safety profile. The additional benefit of developing antibodies against different molecules is their use as specific reagents to determine and understand different protein functions related to specific cell activities. In this study mAb 1479 was described to specifically target the cleavable JM-a isoform on the extracellular domain IV. This domain is susceptible to proteolytic cleavage by TACE, an important step for the initiation of RIP and nuclear signaling of the ErbB4 ICD. Similarly, the binding site of trastuzumab is located in a region of ErbB2 that is susceptible to proteolytic cleavage and trastuzumab has been reported do inhibit ErbB2 ectodomain shedding by ADAM10 (Liu et al 2006, Molina et al 2001). Indeed, when the activities of mAb 1479 were studied, ErbB4 shedding and the

formation of the 80 kDa ErbB4 species was decreased. Additionally mAb 1479 was demonstrated to decrease NRG-1 stimulated phosphorylation of ErbB4 and induce ubiquitination and degradation of ErbB4. Although, mAb 1479 does not interfere with ligand binding to ErbB4 it may sterically prevent TACE-cleavage as well as the formation of active dimers that ultimately result in the inhibition of asymmetric allosteric activation of the kinase domains. Recently a similar mechanism of action was demonstrated for trastuzumab in disrupting the formation of active ErbB2/ErbB3 dimers (Junttila et al 2009). mAb 1479 also increases targeting of ErbB4 to degradative pathways ending up in reduced levels of ErbB4 on the cell membrane. In fact, tumor-inhibitory antibodies targeting ErbB2 have also been reported to induce internalization and degradation of ErbB2 by recruiting c-Cbl and enhancing receptor ubiquitination (Klapper et al 2000).

The benefit of mAb 1479 for breast cancer treatment remains unclear until clinical verification. However, ErbB4 is frequently present in various other cancer tissues (Ljuslinder et al 2009, Rickman et al 2009, Xu et al 2008, Zeng et al 2008), and experimental downregulation of ErbB4 in different tumor cells suppresses growth (Prickett et al 2009, Tang et al 1999). A monoclonal antibody targeting ErbB4 (MAb-3 (clone H.72.8)) has been reported to have growth-suppressing activities of lung (Starr et al 2006) and prostate cancer (Vexler et al 2008) cells. Also, recent findings about activating somatic mutations of ErbB4 in metastatic melanoma (Prickett et al 2009) propose a causal role of ErbB4 in carcinogenesis and supports the development of tools, such as ErbB4 antibodies, to target ErbB4 in cancer (Kurppa and Elenius 2009).

6.2 ErbB4 shedding in cancer

ErbB4 ectodomain shedding was increased in breast cancer tissue compared to normal mammary epithelium and targeting of ErbB4 cleavage with mAb 1479 demonstrated effective anti-tumor activity both *in vitro* and *in vivo*. The increased shedding of ErbB4 was initially hypothesized to associate with increased accumulation of the cytosolic ErbB4 receptor fragment (m80 kDa) because a significant amount of the 80 kDa cleavage product was seen to accumulate in the xenograft tumors. However, the reason for high accumulation of the 80 kDa product *in vivo* seemed to be a result of decreased degradation of ErbB4 rather than increased ectodomain shedding. Thus, nuclear accumulation of ErbB4 ICD *in vivo* does not provide information about increased ErbB4 cleavage.

Consistent with the predominant regulation of the 80 kDa fragment at the level of stability, serum ErbB4 ectodomain levels were not associated with nuclear ErbB4 immunoreactivity. Neither were ErbB4 ectodomain levels associated with total ErbB4 expression by immunohistochemistry. These findings suggest

that ErbB4 shedding does not reflect total ErbB4 expression but rather the activity of ErbB4-sheddases, such as TACE, within the tumor sample. In accordance, previous reports have demonstrated heterogeneity of TACE expression in breast cancer (Borrell-Pages et al 2003, Määttä et al 2006). Similarly, high levels of serum ErbB2 do not consistently associate with ErbB2 overexpression (Leary et al 2009). However, high level of the shed ErbB2 ectodomain in patient serum has been proposed to decrease sensitivity to trastuzumab by neutralizing and preventing it from reaching the target tissues (Brodowicz et al 1997). Thus, ELISAs measuring ErbB2 ectodomain level have been evaluated as possible predictive tools for trastuzumab response (Leary et al 2009).

The ELISA method for assessing ErbB4 content is quantitative and based on the specific identification of ErbB4 by two different antibodies. The main problem in this assay is that, although specific, the origin of the detected protein cannot be identified. This issue becomes significant if other tissues also express the analyzed protein. For example, the ErbB4 JM-a isoform is commonly expressed in breast cancer tissues and undergoes shedding. However, normal kidney tissue also demonstrates considerable amount of ErbB4 shedding. In contrast to ELISA, the specific location of ErbB4 expression by IHC can be well described, but this method is typically less specific and susceptible to interpretation. In fact, clinical studies assessing the significance of ErbB4 in cancer by IHC show a wide variation of ErbB4 expression levels with different clinicopathological characteristics (Hollmén and Elenius 2010). This contradictory may partly result of false positive or negative staining, different handling of patient material and interpretation as well as different expression and processing of the ErbB4 isoforms.

In conclusion, cancers that overexpress the cleavable ErbB4 isoform may be potential candidates for mAb 1479 therapy. Clinical trials assessing the significance of ErbB4 expression and shedding by IHC and ELISA will be needed to address their predictive and prognostic potential for cancer therapy.

6.3 Targeting gastric cancer with trastuzumab

ErbB2 gene amplification is an important biomarker that identifies patients eligible for trastuzumab treatment. Reports of ErbB2 overexpression in cancers of ovary, endometrium, salivary gland, lung, esophagus and stomach (Ménard et al 2001, Scholl et al 2001) elucidate the potential of expanding trastuzumab treatment also for these diseases. Whether these cancers are clinically sensitive to trastuzumab depends on their gene amplification status that is commonly detected by two standard methods, IHC and FISH. IHC analysis is widely used as the first method to assess ErbB2 overexpression as it is cheap and easy to perform. However, the protocol is susceptible to variations and interpretation.

FISH is less affected by these issues (Ross and McKenna 2001). It is used alone or in parallel with IHC to identify ErbB2 gene amplification. However, in FISH areas of invasive carcinoma may be difficult to identify only by DAPI counterstain. CISH is a modification of FISH also used for the analysis of ErbB2 amplification. There is a strong concordance between FISH and CISH in detecting ErbB2 amplification as most studies report a rate of concordance over 85% (Penault-Llorca et al 2009). The benefit of using CISH over FISH in this study, when staining gastric mucosa, helped to distinguish cancer cells from non-malignant glandular epithelium.

ErbB2 gene was amplified in 12.2% of the studied gastric adenocarcinomas and 24% of the gastroesophageal tumors. The prevalence was similar (8-18% in gastric and 25-32% in gastroesophageal cancer) to what has been detected in other studies using both IHC and FISH (Gravalos and Jimeno 2008, Takehana et al 2002). ErbB2 amplification in gastric cancer was seen to associate with the intestinal histologic type compared to the diffuse type (21.5% vs 2.2%, P = 0.0051). This observation is consistent with other reports (Lin et al 1995, Polkowski et al 1999, Wu et al 1997) and interesting because the subgroup of intestinal type of cancers is usually considered to associate with more favorable outcome than the diffuse type of gastric cancers (Hochwald et al 2000). In fact, ErbB2 amplification was strongly associated with poor carcinoma-specific survival (P = 0.0089) in gastric carcinoma and this association increased significantly in the subgroup of intestinal type of tumors (P = 0.0019). The majority of recently published studies have found ErbB2 amplification to be an important prognostic factor for poor survival in gastric carcinoma (Ross and McKenna 2001). The reason for selective amplification of ErbB2 in intestinal histological type is not known, however this is a feature seen also in breast cancer where ErbB2 amplification is common in invasive ductal carcinomas and uncommon in lobular carcinomas.

Cancers with ErbB2 amplification often benefit from anthracycline-based therapies (Pritchard et al 2006). The mechanism of action of anthracyclines, including doxorubicin and epirubicin, is inhibition of Topoisomerase IIα, which eventually leads to the prevention of DNA replication. Topoisomerase IIα gene is located adjacent to ErbB2 in chromosome 17 and these two genes are commonly co-amplified. Thus, ErbB2 amplification is generally considered as a predictive marker also for anthracycline-based chemotherapy. Analysis of Topoisomerase IIα gene amplification in the gastric adenocarcinoma samples showed that 10 out of 16 cancers with ErbB2 amplification also had Topoisomerase IIα gene amplification. Similarly, co-amplification of these genes was found in 16 out of 24 gastroesophageal carcinomas. These data propose ErbB2 amplification to be a positive marker of anthracycline-response in gastric cancer as well.

The gastric carcinoma cell line with ErbB2 amplification was as sensitive to trastuzumab as the ErbB2 overexpressing breast cancer cell line SKBR-3, a commonly used reference for trastuzumab sensitivity studies (Pegram et al 1999, Normanno et al 2002). Two other studies also report ErbB2 amplification-dependent sensitivity of gastric cancer cells for trastuzumab both *in vitro* and *in vivo* (Matsui et al 2005, Fujimoto-Ouchi et al 2007). These results provide theoretical background for testing trastuzumab clinically in gastric carcinoma patients with amplified ErbB2 either as a single agent or in combination with conventional therapy. In fact, at least three trials are exploring the addition of trastuzumab to chemotherapy in ErbB2-positive gastric or gastroesophageal adenocarcinoma (Gravalos and Jimeno 2008). In the first randomized prospective phase III trial (ToGA) 594 ErbB2-positive patients were randomized to receive standard chemotherapy (cisplatin and capecitabine or 5-fluorouracil) or chemotherapy plus trastuzumab. Trastuzumab treatment increased overall survival from 11.1 to 13.8 months. Although modest, the 2.7-month improvement was clinically significant. The patients who had the greatest benefit were those whose tumors were most strongly ErbB2-positive. In these patients, which included 256 of the total 594 patients overall survival was 17.9 months. In conclusion, trastuzumab is the first targeted agent that shows a survival benefit in gastric cancer.

7 CONCLUSIONS

ErbB receptors are significant players in the initiation and progression of tumorigenesis. The efficient targeting of these receptors by monoclonal antibodies and tyrosine kinase inhibitors has provided new tools for cancer therapy. For the succesful use of these ErbB-targeting drugs, patient selection that is based on identifying specific abnormalities in the activity or expression patterns of ErbB receptors in different cancers is undoubtedly required. For example the anti-ErbB2 (trastuzumab) treatment shows efficacy only in tumors with ErbB2 gene amplification that corresponds to 20-30 percent of all breast cancer patients. ErbB2 gene amplification is observed in other types of cancers as well and this study aimed to identify cancer tissues harboring ErbB2 amplification that show sensitivity to trastuzumab. The intestinal type of gastric cancer demonstrated significant ErbB2 amplification that was associated with trastuzumab sensitivity in the preclinical setting. The increased awareness of the involvement of ErbB2 abnormalities in the intestinal type of gastric cancers has led to the approval of trastuzumab in clinical use also for this disease.

Studies concerning ErbB4 activities in cancer are more difficult to interprete as they infrequently discriminate between the four ErbB4 isoforms that promote growth to different extent. This has resulted in two different hypotheses, one favoring ErbB4 targeting in cancer and one proposing that inhibiting ErbB4 activity is disadvantageous. In any case, if ErbB4 targeting is considered, the specific isoform, cancer type and subpopulation should be well selected. To achieve this, more data on the activities and expression of the different isoforms in cancer tissues is required. To date the cleavable isoform JM-a has demonstrated more oncogenic potential when compared to the non-cleavable JM-b isoform. The JM-a isoform is overexpressed in several cancer tissues and its cleavage seems to be associated with unfavorable effects. This study aimed to develop monoclonal antibody-based tools to understand and target the JM-a isoform and its cleavage in cancer, identify druggable tissues for ErbB4 mAbs and develop predictive biomarkers for ErbB4 mAb treatment. In conclusion, targeting the JM-a isoform by inhibiting its cleavage and subsequent receptor activation shows significance in preventing breast cancer cell growth. Potential ErbB4 inhibiting therapy could optimally be targeted to patients who overexpress ErbB4 and demonstrate ErbB4 cleavage in their cancer tissues.

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