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Marika Koivu

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To my mom.

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ABSTRACT

Predictive biomarkers, such as genetic alterations, are used in personalized cancer medicine to target treatment. Genes encoding members of the ERBB family of receptor tyrosine kinases are well known to harbor genetic aberrations that can drive cancer. These activating gene amplifications or mutations in the tyrosine kinase domain make these receptors potential targets for drugs, such as antibodies or tyrosine kinase inhibitors. Subsequently, several drugs targeting these receptors have been approved for clinical use. In an unselected patient population, the response rate to targeted therapy remains suboptimal, and the development of treatment resistance is essentially inevitable. Thus, there is a need to identify new biomarkers that predict drug responses more efficiently. However, the identification of new predictive mutations among the thousands of mutations discovered in patients requires resources. Several genomic studies and clinical trials have been carried out to provide functional information about the tumors. However, these efforts have highlighted the significant heterogeneity of tumors, and future work is required to take advantage of the full potential of these data.

The aim of this thesis was to screen for predictive *ERBB* mutations among the thousands of theoretically possible genetic alterations. This work presents the results of two screens with different setups. The first screen took advantage of publicly available cancer cell line databases that contain sequencing and drug response data for *ERBB* mutated cancer cell lines. For the second approach, the *in vitro* screen for activating mutations (iSCREAM) platform, previously developed in our laboratory, was modified to allow unbiased simultaneous analysis of thousands of activating mutations in ERBB3. Altogether, 79 potentially actionable *ERBB* mutations were identified. Detailed structural, biochemical, and functional analyses validated six of these mutations as novel activating *ERBB* variants with potential predictive value.

These results demonstrate that there are uncharacterized actionable *ERBB* mutations that can be identified with high-throughput screens. The mutations identified here were distributed across all four ERBB receptors and exhibited different gain-of-function mechanisms. The presence of multiple mutations in our screens also emphasizes the complexity of mutational profiles and that co-occurring mutations may promote additive functional effects.

KEYWORDS: biomarker, cancer, EGFR, ERBB2, ERBB3, ERBB4, mutation, predictive

TURUN YLIOPISTO
Lääketieteellinen tiedekunta
Biolääketieteen laitos
Lääketieteellinen biokemia ja genetiikka
MARIKA KOIVU: Uusien ennustavien *ERBB*-mutaatioiden tunnistaminen kohdennettua syöpähoitoa varten
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TIIVISTELMÄ

Lääkevastetta ennustavia biomarkkereita, kuten geneettisiä muutoksia, käytetään kohdennetussa syöpähoidossa ohjaamaan lääkkeiden määräämistä. Reseptorityrosiinikinaaseihin kuuluvat ERBB-reseptorit ovat tunnettuja onkogeenejä, joiden geneettiset muutokset voivat aiheuttaa syöpää. ERBB-geenien monistumat tai aktivoivat mutaatiot ovat tehneet näistä reseptoreista myös kiinnostavia lääkevaikutuskohteita. Kliinisessä käytössä onkin useita syöpälääkkeitä, kuten vasta-aineita tai tyrosiinikinaasi-inhibiittoreita, jotka kohdistuvat näihin reseptoreihin. Potilaista kuitenkin vain osa saa lääkevasteen kohdennetussa syöpähoidossa ja lääkeresistenssi kehittyy käytännössä kaikille. Siksi tarvitaankin uusia biomarkkereita, jotka kykenevät ennustamaan lääkevasteita entistä tarkemmin. Näiden löytäminen tuhansien mahdollisten mutaatioiden joukosta on kuitenkin haastavaa ja vaatii runsaasti resursseja. Useat genomiset tutkimukset ja kliiniset kokeet, joissa on sekvensoitu kasvaimia sekä kerätty lääkevastedataa, lisäävät tietoa kasvainten molekulaarisista ominaisuuksista ja lääkeherkkyyksistä. Lisää tutkimusta kuitenkin vaaditaan, jotta tätä tietoa voidaan hyödyntää tehokkaasti.

Tämän väitöskirjan tarkoituksena oli tunnistaa uusia ennustavia biomarkkereita tutkimalla ERBB-reseptorien mutaatioita kahdella eri lähestymistavalla. Ensimmäisessä työssä uusia aktivoivia ja lääkevastetta ennustavia *ERBB*-mutaatioita etsittiin julkisesti saatavilla olevista syöpäsolujen tietokannoista, jotka sisältävät sekvensointi- ja lääkevastetietoa *ERBB*-mutatoiduille syöpäsoluille. Toisessa työssä kehitimme edelleen iSCREAM (*in vitro* screen for activating mutations) -menetelmää, mahdollistaen tuhansien ERBB3-reseptorin mutaatioiden samanaikaisen analyysin. Yhteensä 79 mahdollista aktivoivaa *ERBB*-mutaatiota tunnistettiin. Rakennebiologiset, biokemialliset ja toiminnalliset analyysit varmistivat kuuden aikaisemmin tuntemattoman ja mahdollisesti lääkevastetta ennustavan *ERBB*-mutaation löytymisen.

Nämä tulokset osoittavat, että uusia aktivoivia ja ennustavia *ERBB*-mutaatioita on mahdollista löytää suurikapasiteettisella, samaan aikaan tuhansia vaihtoehtoja analysoivalla tutkimuksella. Tutkimuksessa tunnistetut mutaatiot löytyivät eri kohdista ERBB-reseptorien rakenteita ja niillä osoitettiin olevan erilaisia toimintamekanismeja. Useiden samanaikaisten ERBB-mutaatioiden löytyminen osoitti myös, että yhtä aikaa esiintyvillä ERBB-geenien mutaatioilla voi olla toiminnallista merkitystä.

AVAINSANAT: EGFR, ennustava biomarkkeri, ERBB2, ERBB3, ERBB4, mutaatio, syöpä

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Abbreviations

ADC	Antibody-drug conjugate
ALCL	Anaplastic large cell lymphoma
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
AR	Amphiregulin
APC/C	Anaphase-promoting complex/cyclosome
AUC	Area under the curve
BTC	Betacellulin
CCLE	Cancer Cell Line Encyclopedia
CCS	Circular consensus sequencing
CHIP	Carboxyl-terminal HSP70-interacting protein
CLL	Chronic lymphocytic leukemia
CML	Chronic myelogenous leukemia
COSMIC	Catalogue of Somatic Mutations in Cancer
CRC	Colorectal cancer
CS	Cell sorting
cSCC	Cutaneous squamous cell carcinoma
CST	Cell Signaling Technology
CTC	Circulating tumor cell
ctDNA	Cell-free circulating tumor DNA
CTRP	Cancer Therapeutics Response Portal
DLBCL	Diffuse large B cell lymphoma
ECD	Extracellular domain
EGF	Epidermal growth factor
EPG	Epigen
EPR	Epiregulin
FDA	Food and Drug Administration
GAB1	GRB2-associated binding protein 1
GBM	Glioblastoma multiforme
GDSC	Genomics of Drug Sensitivity in Cancer
GIST	Gastrointestinal stromal tumor

GRB2	Growth factor receptor bound protein 2
HB-EGF	Heparin-binding EGF-like growth factor
HNSCC	Head and neck squamous cell carcinoma
IgG1	Immunoglobulin G1
IL3	Interleukin 3
IP	Immunoprecipitation
iSCREAM	*In vitro* screen for activating mutations
JAK	Janus kinase
JM	Juxtramembrane
MAPK	Mitogen-activated protein kinase
MDS	Molecular dynamics simulation
mEC50	Modelled EC50
mTOR	Mammalian target of rapamycin
mTORC2	Mammalian target of rapamycin complex 2
NEDD4	Neural precursor cell expressed developmentally downregulated protein 4
NGS	Next-generation sequencing
NRDP1	Neuregulin receptor degradation protein-1
NRG	Neuregulin
NSCLC	Non-small cell lung cancer
PDK1	Phosphoinositide-dependent protein kinase 1
PDX	Patient-derived tumor xenograft
PFS	Progression-free survival
Ph+	Philadelphia chromosome-positive
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol 3,4,5-trisphosphate
PTB	Phosphotyrosine-binding
PTEN	Phosphatase and tensin homolog
PTP	Protein tyrosine phosphatase
RTK	Receptor tyrosine kinase
rAUC	Relative area under the curve
SH2	Src-homology 2
SNP	Single nucleotide polymorphism
SOCS-5	Suppressor of cytokine signaling-5
SOS	Son of sevenless
SRC	Rous sarcoma virus protein tyrosine kinase
STAT	Signal transducer and activator of transcription
STR	Short tandem repeat
TACE	TNFα-converting enzyme

TGFα	Transforming growth factor-α
TKD	Tyrosine kinase domain
TKI	Tyrosine kinase inhibitor
TRK	Tropomyosin receptor kinase
UIM	Ubiquitin-interacting motif
WB	Western blot

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Koivu MKA, Chakroborty D, Tamirat MZ, Johnson MS, Kurppa KJ, Elenius K. Identification of predictive *ERBB* mutations by leveraging publicly available cell line databases. *Mol Cancer Ther,* 2021; 20 (3): 564–576.

II Koivu MKA, Chakroborty D, Airenne TT, Johnson MS, Kurppa KJ, Elenius K. Trans-activating mutations of the pseudokinase *ERBB3*. Manuscript, 2022.

1 Introduction

Cancer remains the second most common cause of death in the world after cardiovascular diseases (www.ourworldindata.org). Although treatment options for cancer have increased significantly in the last decades due to the identification of common cancer driver genes, suppressor genes, and targetable activating mutations, there are still many patients that eventually develop resistance to treatment or do not respond to cancer drugs at all (Forbes *et al.*, 2009; Stratton, Campbell and Futreal, 2009; Cerami *et al.*, 2012; Lawrence *et al.*, 2013; Min and Lee, 2022). The response rate to targeted therapy in an unselected patient population is low, but the reported median response rates from the Food and Drug Administration (FDA) approval notifications for genome-targeted therapy have increased from 19% to 63.5% between the years 2006 and 2020 (Huang *et al.*, 2014; Haslam, Kim and Prasad, 2021). However, the clinical responses are still usually short-lived and almost always followed by disease progression (Sergina *et al.*, 2007; Engelman and Settleman, 2008; Herter-Sprie, Greulich and Wong, 2013; Kandoth *et al.*, 2013). There are several drugs available that target either a single or multiple proteins simultaneously or target the mutated form of the protein. Tumors are highly complex and heterogeneous, and the frequent occurrence of resistance reflects the need for progress in personalized medicine and biomarker discovery (Chang *et al.*, 2016). Genomic studies and clinical trials highlight the complexity of tumors but will eventually provide important information about targetable biomarkers. As the disease progresses, the uncontrollably dividing cell is more susceptible to a higher incidence of genetic instability and to more genetically divergent tumor cell clones arising in a tumor.

The ERBB family of receptor tyrosine kinases (RTK) are oncogenes that were originally identified in the 1980s and 1990s. The family involves four members: EGFR/ERBB1, ERBB2/HER2/Neu, ERBB3/HER3, and ERBB4/HER4. The protein family is important in normal developmental processes and cell growth, but abnormal signaling of the protein family is well known for its ability to cause cancer (Arteaga and Engelman, 2014). The protein family is commonly known for ERBB2 amplification that drives 15–20% of breast cancers (Slamon *et al.*, 1987) and the activating EGFR mutations in 10–20% of Caucasian and up to 50% of Asian non-

small cell lung cancer (NSCLC) patients (Lynch *et al.*, 2004; Rosell *et al.*, 2009; Zhou *et al.*, 2009; Collisson *et al.*, 2014; Shi *et al.*, 2014). There are several FDA-approved drugs available that target these receptors (Sequist *et al.*, 2013; Jänne *et al.*, 2015; Chan *et al.*, 2016; Wu *et al.*, 2017), but the treatment still faces the same limitations as for other cancer types: acquired resistance and initial non-responsiveness.

The aim of this thesis was to use two different approaches to identify novel activating *ERBB* mutations that predict response to ERBB-targeted drug treatments. These methods included a cancer cell line database screen using three publicly available databases to identify drug-sensitive cell lines harboring an *ERBB* mutation and an *ERBB3 in vitro* screen for activating mutations (iSCREAM) platform of an unbiased screen of randomly mutated *ERBB3* variants that could potentially also act as predictive mutations for ERBB-targeted drug treatment.

2 Review of the Literature

2.1 Biomarkers

2.1.1 Biomarker classes and characteristics

Biomarkers are measurement variables associated with disease outcomes (Ballman, 2015). Several variables can act as a biomarker. For example, body measurements and metabolites, the expression level of a protein, a specific mutation, a specific antigen level, or antibodies can act as biomarkers for various purposes.

Measurement data are increasing constantly in the field of medicine, giving the potential for biomarkers to be taken in for clinical use. Generally, a biomarker must be validated before it can be approved. The only exception to this is with accelerated approval pathways where there are no effective therapies currently available (*Fast Track, Breakthrough Therapy, Accelerated Approval, Priority Review | FDA*, 2018). In an accelerated approval pathway, the use of a surrogate endpoint marker that is thought to predict clinical benefit permits earlier access for patients to a novel therapy. However, the clinical benefit must still be confirmed with clinical studies.

Biomarkers can be classified into several categories, including diagnostic, monitoring, pharmacodynamics/response, safety, prognostic, or predictive biomarkers. Diagnostic biomarkers are markers that can be detected, and that confirm the presence of a disease or a condition (FDA-NIH Biomarker Working Group, 2016). Diagnostic biomarkers are also used to subtype diseases. Diagnostic biomarkers must have low false-positive rates (*e.g.* cancer diagnosis) and low false-negative rates (*e.g.* repeated measurements of blood pressure for hypertension diagnosis).

Monitoring biomarkers are markers that can be measured serially and that can be used to monitor the status of a disease or a medical condition. Important examples of monitoring biomarkers in clinical use are low-density lipoprotein in cholesterol-lowering drug use or the CD4 counts in HIV infection treatment (Califf, 2018). Monitoring biomarkers are also important in clinical studies where they can be used to monitor, for example, toxicities, therapeutic responses, and complications of a disease or a given therapy.

Pharmacodynamic/response biomarkers measure the change of a biomarker level in response to a medical product or an environmental agent (Califf, 2018). These biomarkers are useful in both clinical trials and therapeutic development, and in clinical practice where one can monitor whether the drug has an effect.

Safety biomarkers are markers that are used to measure the likelihood, presence, or extent of toxicity. Adverse effects from a specific treatment can be identified from safety biomarkers. Safety biomarkers are commonly measured before or after exposure to a medical intervention or an environmental agent (Califf, 2018).

Prognostic biomarkers are markers that inform, for example, a likely cancer outcome, regardless of the received treatment. Prognostic biomarkers can also be used to identify the likelihood of disease recurrence or progression. Prognostic biomarkers can be mutations in a specific cancer type that predict worse progression-free survival (PFS). Prognostic biomarkers should be differentiated from susceptibility/risk biomarkers that indicate the potential for developing a medical condition or a disease of those who do not currently have any apparent disease or medical condition (FDA-NIH Biomarker Working Group, 2016).

Predictive biomarkers are markers that predict response to treatment. These can be both favorable or unfavorable effects from exposure to a medical product or environmental agents (FDA-NIH Biomarker Working Group, 2016). A predictive biomarker can be a mutation that makes a specific tumor more responsive to a targeted treatment. Gefitinib in the treatment of NSCLC patients is an excellent example of a drug targeting a predictive biomarker, as patients harboring the mutated form of EGFR in their tumors are more responsive to gefitinib as compared to those harboring EGFR wild-type (Lynch *et al.*, 2004; Rosell *et al.*, 2010). It is also possible for a biomarker, for example ERBB2, to be prognostic and predictive at the same time (Masood and Bui, 2002; Petrelli and Barni, 2012). This study uses the term predictive mutation also to describe a mutation that is growth-activating and responsive to targeted drugs in a cell line.

2.1.2 Precision oncology and the underlying challenges

Early detection of cancer and early treatment can save millions of cancer patients from premature death (World Health Organization, 2007). There is a need for new biomarkers for precision oncology, but also for high-quality diagnostics tests and their corresponding technical and clinical validation (Lawrence *et al.*, 2013; Hyman, Taylor and Baselga, 2017). However, there are still challenges in identifying new biomarkers and drug targets. These challenges include the limited amount of data available from genomes and biological processes of cancer patients, lack of suitable assays and analysis methods, and drug target discovery being very expensive and time-consuming and having a great risk for failure. Deep sequencing has also

revealed subclinical mutations that are found in a small subclone of the tumor. These can be later found to be more clinically relevant, but our knowledge of oligoclonal heterogeneity and clonal selection is still limited (Anderson *et al.*, 2011; Gerlinger *et al.*, 2012; Su *et al.*, 2012). These findings can affect, for example, biopsy strategies if there are different secondary mutations or other changes in metastatic tumor tissues that affect treatment options.

2.1.2.1 Challenges in data analysis and validation

Patient studies that sequence patient tumors and monitor treatment responses have multiplied in a short amount of time. The huge amount of sequencing data makes determining connections, analyzing the functional role of the data, and understanding their molecular mechanisms of action challenging. The data are complex, and the communication between cancer genomics and cancer biology is challenging. Experimental validation is another bottleneck, as all candidates must be validated before they can be turned into therapeutic targets or biomarkers. Validation is time-consuming and takes a lot of resources. *In vitro* methods for validation are high-throughput and well-suited for first-pass validation as they can be more easily used for screening a large number of genomic candidates. However, they do not always offer the same predictive information as *in vivo* assays (Chin and Gray, 2008; Chin, Andersen and Futreal, 2011). *In vivo* assays are more reliable for making real conclusions about the study targets but require more labor, time, and resources (Chin, Andersen and Futreal, 2011). Thus, systematic assays that try to identify targets more reasonably and cost-effectively are needed. The immense amount of sequencing data has identified a number of somatic mutations that are located in potentially actionable cancer genes, but these do not have any biological or clinical validation. This means that the knowledge gap in situations like this impairs the ability to fully take advantage of these data.

It is also important to have sample data available about tumors prior to treatment and normal-matched samples, as using only data from patients' samples with prior therapies means that many of the mutations detected are those that arise only upon selective pressure from therapeutic treatment intervention. Sample processing is also an important factor, as formalin-fixed or paraffin-embedded fragments can alter nucleic acids. Low tumor content percentage in a sample can also decrease test sensitivity or introduce false-positive mutation calls (Moorcraft, Gonzalez and Walker, 2015). Liquid biopsies, simple blood samples for example, would be ideal as a minimally invasive method to detect and monitor disease characteristics and progression. Liquid biopsies could be used in repeated sampling, and the circulating tumor cells (CTCs) and/or cell-free circulating tumor DNA (ctDNA) could offer

more information about the tumor (Heitzer *et al.*, 2013; Bettegowda *et al.*, 2014; Collins *et al.*, 2017).

2.1.3 Cancer cell line panels and patient-derived screens

There have been numerous efforts in trying to generate datasets that could accelerate biomarker identification. Examples of these large datasets containing thousands of cancer cell lines, their sequencing data, and therapeutic responses to a variety of compounds include the Cancer Cell Line Encyclopedia (CCLE) (Barretina *et al.*, 2012), the Cancer Therapeutics Response Portal (CTRP) (Seashore-Ludlow *et al.*, 2015), and the Genomics of Drug Sensitivity in Cancer (GDSC) (Yang *et al.*, 2013). These datasets are freely available with the purpose to share knowledge and to improve biomarker target identification.

Large-scale cell line panels are useful in covering low-frequency cases of genetic aberrations and performing experimental processing. However, they do not capture the intratumoral heterogeneity of human tumors in the *in vivo* environment (Huang *et al.*, 2014). Addressing this shortcoming, patient-derived genomic screens such as MSK-IMPACT (Cheng *et al.*, 2015), AACR Project GENIE (Sweeney *et al.*, 2017), and many more offer sequencing and patients' treatment response data. However, the generation of data of this magnitude requires a lot of time and resources.

Patient-derived tumor xenografts (PDXs) are also providing solutions to these shortcomings as they are cellularly heterogeneous, molecularly diverse, and the histology is as seen in patient tumors. PDXs are biologically stable, considering global gene expression patterns, mutational status, metastatic potential, drug responsiveness, and tumor architecture. However, they do not represent the human microenvironment, and because they are used in immune-compromised rodents, they do not recapitulate the immune systems (Huang *et al.*, 2014).

2.1.4 Biomarker studies in the clinic

Genomic profiling is becoming routine across multiple cancer types, and clinical trials are more often driven by biomarkers. Biomarker clinical trials are divided into different branches. A master protocol can be considered to be on top and to refer to a single, overarching design that is developed to test multiple hypotheses (Redman and Allegra, 2015; Woodcock and LaVange, 2017; Park *et al.*, 2020). Master protocols are usually classified into umbrella trials, basket trials, and platform trials. In a basket trial, targeted therapy is evaluated for multiple diseases that share a common molecular alteration or a risk factor (Park *et al.*, 2019). In an umbrella trial, a single disease can be divided into multiple subgroups based on different molecular or other predictive risk factors, and each of these subgroups receives a different

targeted therapy (Park *et al.*, 2019). Platform trials are multi-arm, multi-stage design trials that evaluate several interventions against a common control group (Berry, Connor and Lewis, 2015). There are currently several basket and umbrella trials ongoing (clinicaltrials.gov). Basket trials leading to FDA approval include vemurafenib for BRAF V600E mutated melanoma (Robert *et al.*, 2015) and larotrectinib for tropomyosin receptor kinase (TRK) fusion-positive cancers (Drilon *et al.*, 2018). Clinical trials have also moved more towards basket trial designs as new potential driver mutations are identified.

Challenges in identifying specific predictive biomarkers include that nearly all of the genomic alterations currently used to guide targeted therapy selection also occur across a variety of other cancer types (Kandoth *et al.*, 2013; Chang *et al.*, 2016). A textbook example of this is an alteration in BRAF residue V600 that is found in cutaneous melanoma and targeted by RAF and MEK inhibitors such as vemurafenib and dabrafenib (Robert *et al.*, 2015). However, the same alteration is also found in non-melanoma cancers, and in those cancer types, benefit from the same treatment is not always observed (Hyman *et al.*, 2015).

A biomarker needs to be identified from a patient with specificity. Companion biomarkers help select and guide treatment options for patients. They are used together with therapy to predict likely response or toxicity (Duffy and Crown, 2013). Not being able to specifically detect a biomarker that should guide targeted therapy, severely restricts the use of a therapy. Examples of companion biomarkers or diagnostics include the detection of *ERBB2* amplification from breast cancer patients with immunohistochemistry or fluorescent in situ hybridization (anti-ERBB2 therapy) (Wolff *et al.*, 2007; Hammond *et al.*, 2010), detection of *KRAS* mutations from colorectal cancer patients with sequencing methods (anti-EGFR therapy) (Bardelli and Siena, 2010), and the detection of *BRAF* V600E mutation from melanoma patients with, for example, the cobas 4800 BRAF V600 Mutation Test (Menzies *et al.*, 2012). Developing a drug along with its companion diagnostic would be the most beneficial method to obtain a biomarker that can also be detected with a validated test. In order for the companion diagnostic to be reliable in the clinic, the analytical and clinical validation needs to be consistent, and similar results should be obtained between institutions (Schilsky *et al.*, 2012; Williams *et al.*, 2012).

2.1.5 Targeted drugs

Despite all the challenges in identifying new biomarkers and drug targets, many drugs have been approved for targeted treatment. The FDA-approved kinase inhibitors in use for cancer treatment are listed in Table 1, and the FDA-approved antibodies for cancer treatment are listed in Table 2.

Table 1. Table of FDA-approved kinase inhibitors for cancers with identified biomarkers. Order by year. Modified from (Roskoski, 2022) table. Abbreviations: ALL, acute lymphoblastic leukemia; CML, chronic myelogenous leukemia; GIST, gastrointestinal stromal tumors; NSCLC, non-small cell lung cancers; Ph+, Philadelphia chromosome-positive.

YEAR	DRUG	PRIMARY TARGET	DISEASE
2021	Infigratinib	FGFR2	Cholangiocarcinoma with FGFR2 fusion proteins
2021	Mobocertinib	EGFR	NSCLC for EGFR-positive exon 21 insertions
2021	Tepotinib	MET	NSCLC with *MET* mutations
2020	Avapritinib	PDGFRα	GIST with *PDGFRα* exon 18 mutation
2020	Capmatinib	MET	NSCLC with *MET* exon 14 skipping
2020	Pemigatinib	FGFR2	Advanced cholangiocarcinoma with a FGFR2 fusion or rearrangement
2020	Pralsetinib	RET	RET-fusion (i) NSCLC, (ii) medullary thyroid cancer, (iii) thyroid cancer
2020	Selpercatinib	RET	RET-fusion NSCLC and thyroid cancers and RET mutant medullary thyroid cancers
2020	Tucatinib	ERBB2/HER2	Combination second-line treatment for HER2-positive breast cancer
2019	Entrectinib	TRKA/B/C, ROS1	Solid tumors with NTRK fusion proteins, ROS1-positive NSCLC
2018	Binimetinib	MEK1/2	*BRAF* V600E/K melanoma (combination therapy)
2018	Dacomitinib	EGFR	*EGFR*-mutant NSCLC
2018	Encorafenib	BRAF	*BRAF* V600E/K melanoma (combination therapy)
2018	Larotrectinib	TRKA/B/C	Solid tumors with NTRK fusion proteins
2018	Lorlatinib	ALK	ALK-positive NSCLC
2017	Brigatinib	ALK	ALK-positive NSCLC
2017	Neratinib	ERBB2/HER2	HER2-positive breast cancer
2015	Alectinib	ALK, RET	ALK-positive NSCLC
2015	Cobimetinib	MEK1/2	*BRAF* V600E/K melanoma (combination therapy)
2015	Palbociclib	CDK4/6	Estrogen receptor- and HER2-positive breast cancer
2014	Ceritinib	ALK	ALK-positive NSCLC resistant to crizotinib
2013	Dabrafenib	BRAF	*BRAF* V600E/K melanoma, BRAF V600E NSCLC and anaplastic thyroid cancer
2013	Trametinib	MEK1/2	BRAF V600E/K melanoma, BRAF V600E NSCLC
2012	Ponatinib	BCR-ABL	Ph^+ CML or ALL
2011	Crizotinib	ALK, ROS1	ALK or ROS1-positive NSCLC

2011	Vemurafenib	BRAF	BRAF V600E melanoma
2007	Lapatinib	EGFR/ERBB2/HER2	HER2-positive breast cancer
2007	Nilitinib	BCR-ABL	Ph^+ CML
2001	Imatinib	BCR-ABL	Ph^+ CML or ALL, aggressive systemic mastocytosis, chronic eosinophilic leukemia, dermatofibrosarcoma protuberans, hypereosinophilic syndrome, GIST, myelodysplastic/ myeloproliferative disease

Table 2. Table of all FDA-approved antibodies for cancer. Order by year. Abbreviations: ALCL, systemic anaplastic large cell lymphoma; ALL, acute lymphocytic leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; cSCC, cutaneous squamous cell carcinoma; DLBCL, diffuse large B cell lymphoma. (Data acquired from fda.gov.)

YEAR	DRUG	PRIMARY TARGET	DISEASE
2022	Relatlimab	LAG-3	Melanoma
2022	Tebentafusp	gp100, CD3	Metastatic uveal melanoma
2021	Tisotumab vedotin	Tissue factor	Cervical cancer
2021	Loncastuximab tesirine	CD19	Diffuse large B-cell lymphoma
2021	Dostarlimab	PD-1	Endometrial cancer
2021	Amivantamab	EGFR, MET	NSCLC with EGFR exon 20 insertion mutations
2020	Margetuximab-cmkb	ERBB2/HER2	HER2+ metastatic breast cancer
2020	Sacituzumab govitecan	TROP-2	Triple-negative breast cancer
2020	Tafasitamab	CD19	DLBCL
2020	Naxitamab-gqgk	GD2	High-risk neuroblastoma and refractory osteomedullary disease
2020	Belantamab mafodotin	B-cell maturation antigen	Multiple myeloma
2020	Isatuximab	CD38	Multiple myeloma
2019	Fam-trastuzumab deruxtecan-nxki	ERBB2/HER2	Breast cancer
2019	Polatuzumab vedotin	CD79b	DLBCL
2019	Enfortumab vedotin	Nectin-4	Urothelial cancer
2018	Cemiplimab	PD-1	cSCC
2018	Moxetumomab pasudotox	CD22	Hairy cell leukemia
2017	Inotuzumab ozogamicin	CD22	ALL
2017	Gemtuzumab ozogamicin	CD33	AML
2017	Durvalumab	PD-L1	Bladder cancer

2017	Avelumab	PD-L1	Merkel cell carcinoma
2016	Atezolizumab	PD-L1	Bladder cancer
2015	Daratumumab	CD38	Multiple myeloma
2015	Elotuzumab	SLAMF7	Multiple myeloma
2015	Dinutuximab	GD2	Neuroblastoma
2015	Necitumumab	EGFR	NSCLC
2014	Blinatumomab	CD19, CD3	ALL
2014	Ramucirumab	VEGFR2	Gastric cancer
2014	Nivolumab	PD-1	Melanoma, NSCLC
2014	Pembrolizumab	PD-1	Melanoma
2014	Alemtuzumab	CD52	Multiple sclerosis; CML
2013	Obinutuzumab	CD20	CLL
2012	Ado-trastuzumab emtansine	ERBB2/HER2	Breast cancer
2012	Pertuzumab	ERBB2/HER2	Breast Cancer
2011	Brentuximab vedotin	CD30	Hodgkin lymphoma, systemic ALCL
2011	Ipilimumab	CTLA-4	Metastatic melanoma
2009	Ofatumumab	CD20	CLL
2006	Panitumumab	EGFR	Colorectal cancer
2004	Bevacizumab	VEGF-A	Colorectal cancer
2004	Cetuximab	EGFR	Colorectal cancer
2002	Ibritumomab tiuxetan	CD20	Non-Hodgkin lymphoma
1998	Trastuzumab	ERBB2/HER2	Breast cancer
1997	Rituximab	CD20	Non-Hodgkin lymphoma

2.2 Genetic alterations

2.2.1 Genomic instability and alteration types in cancer

Cells have several mechanisms in cell cycle and replication processes that prevent genomic instability. These include telomere function, centrosomes, the spindle assembly checkpoint (delay of cell cycle progression and attachment error correction), epigenetic modifications, and excision repair pathways (including nucleotide or base excision repair, DNA mismatch repair, and double-strand break repair) (Ferguson *et al.*, 2015). However, malfunction of these mechanisms can result in genomic instability and alterations. Catastrophic events such as

chromoplexy, kataegis, or chromothripsis can cause multiple mutations or substantial reconfigurations of the genome in one somatic mutational process (Berger *et al.*, 2011; Stephens *et al.*, 2011; Nik-Zainal, Alexandrov, *et al.*, 2012; Nik-Zainal, Van Loo, *et al.*, 2012; Rausch *et al.*, 2012; Roberts *et al.*, 2012; Baca *et al.*, 2013; Korbel and Campbell, 2013).

DNA sequence variations include single nucleotide polymorphisms (SNPs), deletions, insertions, and short tandem repeats (STRs), where SNPs are the most commonly used type of DNA variation in biomarker applications. Somatic mutations are mutations that are found in any non-germ cell of the organism and can lead to tumorigenesis. Germline mutations are mutations already in a body's reproductive cell and thus present in all cells in the body as they are inherited from the parents. In relation to cancer, there are several different types of mutations, and they are listed in Table 3.

Table 3. Table of different types of genetic alteration.

GENETIC ALTERATION TYPE	MEANING
Synonymous mutation	A mutation that does not alter the encoded amino acid.
Nonsynonymous mutation	A mutation that alters the amino acid sequence so that a mutant version is produced.
Missense mutation	A single-nucleotide substitution that results in an amino acid substitution.
Nonsense mutation	A single-nucleotide substitution that results in a stop codon.
Passenger mutation	A mutation that has no functional role (wild-type like) and does not possess growth advantage.
Driver mutation	A mutation that confers for a selective growth advantage of the cell and drives the development of cancer.
Hotspot mutation	A cancer mutation that is recurrently observed among patients and is more likely a functional mutation.
Gatekeeper mutation	A way for a cancer cell to escape treatment.
Indel	A mutation of a small insertion or deletion.
Homozygous deletion	Deletion of both copies of a gene segment.
Gene amplification	An increase in the number of copies of a gene in a genome.
Gene fusion	Parts of two different genes are joined when part of the DNA from one chromosome moves to another chromosome.
Translocation	A type of rearrangement where regions from two nonhomologous chromosomes are joined.

2.2.2 Tumor evolution

Tumor evolution is seen to be driven either by an evolutionary Darwinian model or by a cancer stem cell model. The acquired mutations gaining survival advantage can be seen in the Darwinian model to arise from biologically similar tumor cells that have equal opportunities to acquire mutations and spawn new subclones (Hanahan and Weinberg, 2011). The cancer stem cell theory suggests that there are ancestors of a population of more differentiated cells that have limited proliferative capacities. This can be seen as a strictly hierarchical model or a non-hierarchical model where the cancer stem cells either are a biologically distinct population and the only ones capable of self-renewing and tumorigenesis or that potentially every tumor cell has plasticity and has the potential to de-differentiate and react to intrinsic or microenvironmental factors (Chaffer and Weinberg, 2015).

Tumors become malignant lesions when mutations are acquired over time (Tomasetti, Vogelstein and Parmigiani, 2013). The first step for a cell to become malignant involves a gatekeeper gene mutation. This mutation provides a growth advantage over normal cells (Kinzler and Vogelstein, 1997). However, tumor suppressor genes regulate cell divisions and keep cells from excessive growth unless they get inactivated by mutations. Additional mutations acquired over time can make the tumor become metastatic and invade distant organs (Nowell, 1976; Fearon and Vogelstein, 1990).

Cancer genome analysis research has shown that tumor types have different amounts of mutations (Garraway and Lander, 2013). Division time of the cell type (*e.g.* colon epithelial cells versus brain glial cells) varies substantially and has a clear effect on how many mutations are acquired over time. For example, lung tumors and melanomas have about 200 mutations per tumor, derived from ultraviolet light or cigarette carcinogens (Vogelstein *et al.*, 2013). On the other hand, pediatric tumors and leukemia have far fewer mutations (about ten mutations per tumor). On average, 2.6 coding point driver mutations can be found in tumors (Martincorena *et al.*, 2017; Campbell *et al.*, 2020).

The implementation of next-generation sequencing (NGS) and the advent of the genomic era in cancer research have enabled that mutational landscapes have been established in almost all types of human tumors (Vogelstein *et al.*, 2013). The best known hotspot mutations include mutations in genes *BRAF*, *PIK3CA*, *TP53*, and *KRAS*. However, 85% of all hotspot mutations are mutated in less than 5% of tumors of all cancer types, supporting the long tail of the frequency distribution of somatically mutated genes (Garraway and Lander, 2013; Chang *et al.*, 2016). Many of the identified hotspot mutations are very rare and can occur only in few samples. These mutations may never be found in patients with minimal frequencies (2–3%) (Chang *et al.*, 2016).

2.2.3 Intratumoral heterogeneity

Tumors can have different genetic heterogeneity, and focusing on only exonic mutations can be problematic as there are also other events affecting cancer initiation and progression (Vogelstein et al., 2013). Acquired driver mutations can favor genetic instability (mutations in DNA-repair genes), resistance to treatments (phosphatase and tensin homolog (PTEN) loss), or immunoescape (Juric et al., 2015; Keenan, Burke and Van Allen, 2019). Subclonality can also arise as the tumor progresses (Gerstung et al., 2020; von Loga et al., 2020). However, the main source for clonal diversity in tumors is the emergence of passenger mutations that have no growth advantage over the driver mutations that confer a selective growth advantage (Flavahan, Gaskell and Bernstein, 2017; Martincorena et al., 2017).

Intratumoral heterogeneity is observed among the cells of one tumor. Intratumoral heterogeneity is common and derived from normal cell divisions. Intratumoral heterogeneity confers plasticity to evolving tumors and drives cancer cell proliferation, resistance to therapy, and invasion (Collins *et al.*, 2017; McGranahan and Swanton, 2017; Dagogo-Jack and Shaw, 2018).

Intermetastatic heterogeneity is observed in different metastatic lesions in the same patient. Intrametastatic heterogeneity is observed among the cells of an individual metastasis. Resistance mutation clones are usually derived within one lesion and are responsible for the treatment not eradicating the entire mass (Komarova and Wodarz, 2005; Durrett and Moseley, 2010; Turke et al., 2010).

2.3 Receptor tyrosine kinases

2.3.1 Receptor tyrosine kinase structure

There are currently 55 different RTKs that can further be grouped into 19 subfamilies based on kinase domain sequence. These subfamilies consist of ALK, AXL, DDR, EGFR, EPH, FGFR, INSR, MET, MUSK, PDGFR, PTK7, RET, ROR, ROS, RYK, STYK1, TIE, TRK, VEGFR (Wheeler and Yarden, 2015). All RKTs share similar structures, including a ligand binding domain in the extracellular region, a single transmembrane helix, and a cytoplasmic region consisting of the juxtamembrane (JM) regulatory region, protein tyrosine kinase domain (TKD) and a carboxy (C-) terminal tail (Lemmon and Schlessinger, 2010). The key components and mechanisms of activation are highly conserved between organisms. RTKs are important in numerous cellular processes, such as the regulation of cell growth, differentiation, and survival (Ullrich J., 1990; Blume-Jensen and Hunter, 2001; Lemmon and Schlessinger, 2010).

2.3.2 Abnormal RTK activation

When RTK signaling is abnormal, due to genetic changes in the receptor that alter the activity or changes in the regulation of RTKs, diseases can develop. These diseases include arteriosclerosis, autoimmune diseases, cancer, diabetes, inflammation, and developmental problems (Lemmon and Schlessinger, 2010).

Human cancer can be derived from four different abnormal RTK activation mechanisms: gain-of-function mutations, gene amplification, chromosomal rearrangements, and/or autocrine activation (Lemmon and Schlessinger, 2010; Du and Lovly, 2018).

Gain-of-function mutations in a RTK lead to activation through aberrant downstream signal transduction. This pathway activation is not subjected to normal balances or checkpoints. The emerging driver mutations confer a selective growth advantage to cells (Vogelstein *et al.*, 2013). It is typical for somatic mutations to cluster in conserved regions of residues, such as the DFG motif located in the kinase activation loop (Lahiry *et al.*, 2010; Medves and Demoulin, 2012).

Overexpression and genomic amplification are other mechanisms for RTK activation. Overexpression has been found in a variety of human tumor types, such as EGFR in glioblastoma multiforme (GBM) (Lopez-Gines *et al.*, 2010), ERBB2 in breast (Yaziji *et al.*, 2004), or MET in lung cancer (Xu *et al.*, 2010). Overexpression causes a local increase in the concentration of the receptor, and this results in increased RTK signaling (Carraway and Sweeney, 2002). Overexpression is mainly derived from gene amplification, but other mechanisms include transcriptional or translational enhancement (Ludes-Meyers *et al.*, 1996; Reznik *et al.*, 2008), derailment of normal regulatory mechanisms (loss of phosphatases) (Sun *et al.*, 2011), or other negative regulators (Mudduluru *et al.*, 2011; Maiti *et al.*, 2013). Gene amplification is derived from increases in the copy number of a specific region of the genome (Albertson, 2006).

The third form of abnormal RTK activation is chromosomal rearrangements. Several chromosomal rearrangements have been identified that lead to the formation of tyrosine kinase fusion oncoproteins (Brennan *et al.*, 2013; Collisson *et al.*, 2014; Stransky *et al.*, 2014). The aberrant fusion proteins are very often therapeutically targetable with small molecule inhibitors, thus making their identification important. The best-known tyrosine kinase fusion protein is the “Philadelphia Chromosome”, a fusion of genes *ABL1* and *BCR*, resulting in BCR-ABL (Diamond, Goldman and Melo, 1995; Nowell, 2007). Fusions among RTKs include ALK fusions (Lovly *et al.*, 2014; Childress *et al.*, 2018) and RET fusions (Dacic *et al.*, 2014; Stransky *et al.*, 2014; Kato *et al.*, 2017).

The fourth mechanism of abnormal RTK activation is autocrine activation which is derived from growth factors or cytokines. Autocrine activation can form a ligand/receptor loop that drives the growth of the cell (Walsh *et al.*, 1991).

2.3.3 Pseudokinases

There are estimates that about 10% of the 518 protein kinases in humans are kinase-dead and lack highly conserved residues important for catalytic activity (Manning *et al.*, 2002; Boudeau *et al.*, 2006; Zeqiraj and van Aalten, 2010). The three motifs essential for catalytic activity include VAIK, HRD, and DFG motifs (Boudeau *et al.*, 2006). VAIK (Val-Ala-Ile-Lys) motif interacts with the α and β phosphates of ATP with the lysine residue (Boudeau *et al.*, 2006). HRD (His-Arg-Asp) motif contains the aspartic acid that functions as the catalytic residue (Boudeau *et al.*, 2006). DFG (Asp-Phe-Gly) motif binds the Mg^{2+} ions with the aspartic acid residue, and these ions coordinate the β and γ phosphates of ATP in the ATP-binding cleft (Boudeau *et al.*, 2006). These residues are known to be essential for catalytic activity based on mutagenesis studies. Pseudokinases lack residues in at least one of these three motifs.

There are eight pseudokinases within RTKs: ERBB3, EphA10, EphB6, PTK7, RYK, ROR1, ROR2, and STYK1. Pseudokinases ERBB3, EphB6, EphA10, and STYK1 are suggested to have intracellular domains that lack catalytic activity (Manning *et al.*, 2002). These pseudokinases lack phosphotransferase activity required for kinase activity due to substitutions of these conserved and essential catalytic residues mentioned above (Manning *et al.*, 2002; Boudeau *et al.*, 2006). The other pseudokinases (PTK7, ROR1, ROR2, and RYK) have substitutions in conserved motifs that make these proteins lack both kinase activity and ATP binding (Katso, Russell and Ganesan, 1999; Manning *et al.*, 2002; Gentile *et al.*, 2011; Murphy *et al.*, 2014). PTK7, ROR1, ROR2, and RYK have also been linked to Wnt-signaling, which has roles in development (Niehrs, 2012; Sheetz *et al.*, 2020). Pseudokinases are presumed to have some key noncatalytic functions, such as functions as docking platforms or scaffolding proteins, structural elements, or regulatory domains (Boudeau *et al.*, 2006; Zeqiraj and van Aalten, 2010).

2.4 *ERBB* gene family

2.4.1 *ERBB* gene family and its importance in developmental processes

The ERBB family of RTKs consists of four different receptor family members: epidermal growth factor receptor (EGFR/ERBB1) encoded by the gene *EGFR*, ERBB2 (HER2/Neu) encoded by the gene *ERBB2*, ERBB3 (HER3) encoded by the gene *ERBB3*, and ERBB4 (HER4) encoded by the gene *ERBB4*. Different isoforms of ERBB receptors can be found in normal and cancer cells (Scott *et al.*, 1993; Elenius *et al.*, 1997, 1999; Lee and Maihle, 1998; Lee *et al.*, 2001; Hua *et al.*, 2020).

The ERBB receptors are expressed in several epithelial, mesenchymal, and neuronal tissues. The ERBB receptors are important in many developmental processes and in proliferation and differentiation of cells (Olayioye *et al.*, 2000). The importance of these receptors is demonstrated as lethality in mice of null mutations in individual *ERBB* loci. Loss of EGFR has been shown to lead to embryonic or perinatal lethality in mice, affecting brain, skin, lung, and gastrointestinal tract development (Miettinen *et al.*, 1995; Sibilia and Wagner, 1995; Threadgill *et al.*, 1995; Sibilia *et al.*, 1998). ERBB2 and ERBB4 are important in heart development, demonstrated by the midgestation lethality of ERBB2 or ERBB4 null mice (Gassmann *et al.*, 1995; Lee *et al.*, 1995). ERBB2 is necessary also in peripheral nervous system development (Morris *et al.*, 1999). ERBB3 knockout mice usually display defective heart valve formation, generalized neural crest defect, and lack of Schwann cell precursors (Erickson *et al.*, 1997; Riethmacher *et al.*, 1997).

2.4.2 ERBB receptor ligands

ERBB ligands can be divided roughly into three groups (Figure 1A). The first group consists of epidermal growth factor (EGF), epigen (EPG), transforming growth factor-α (TGFα), and amphiregulin (AR), which all bind specifically to EGFR. The second group consists of betacellulin (BTC), heparin-binding EGF-like growth factor (HB-EGF), and epiregulin (EPR) that can bind both EGFR and ERBB4 (Harris, Chung and Coffey, 2003). The third group consists of neuregulins (NRG) which can bind only to ERBB4 (NRG-3 and NRG-4) or to both ERBB3 and ERBB4 (NRG-1 and NRG-2) (Riese *et al.*, 1995; Chang *et al.*, 1997; Zhang *et al.*, 1997; Harari *et al.*, 1999; Hynes and Lane, 2005). The expression pattern of ERBB ligands differs organ- and developmental stage-specifically. For example, NRG-1 is widely expressed, while the expression of other neuregulins is more regulated (Meyer and Birchmeier, 1995). NRG-3 is expressed in the developing and adult nervous system, and NRG-4 is found to be highly expressed in the pancreas (Zhang *et al.*, 1997; Harari *et al.*, 1999). The ERBB ligands have also different binding affinities, which affects the signal strength and duration. Another regulation for ERBB ligands is the pH stability of the ligand-receptor interaction. This has been demonstrated to affect receptor trafficking, particularly in lysosomes (French *et al.*, 1995; Waterman *et al.*, 1998).

2.4.3 ERBB receptor structure

The ERBB receptors consist of an extracellular ligand binding domain, a single membrane-spanning region, a tyrosine kinase-containing domain, and a cytoplasmic tail. The extracellular domain (ECD) consists of domains I-IV where domains I and

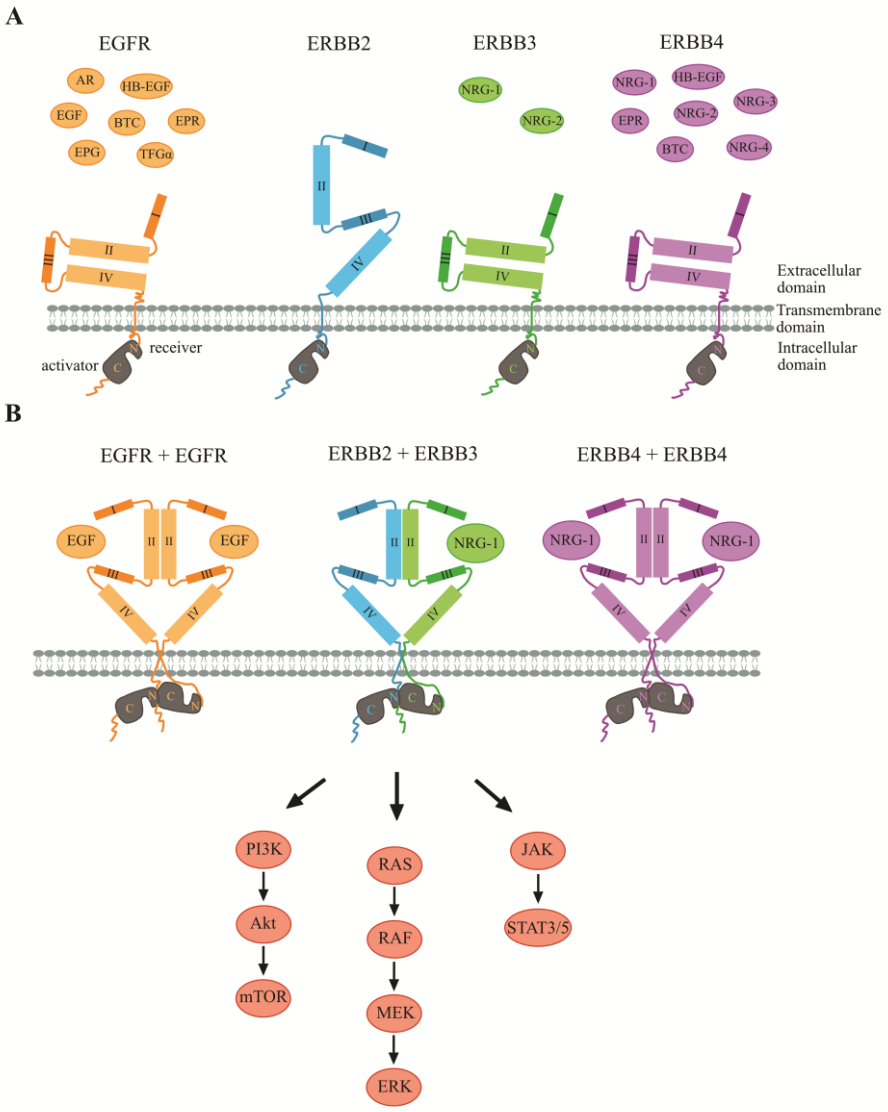

Figure 1. ERBB receptors and their activation. **A,** The ERBB receptors (EGFR, ERBB3, and ERBB4 in a tethered conformation and ERBB2 in a constitutively open conformation) along with their binding ligands. **B,** ERBB receptor dimerization is illustrated as EGFR homodimer, ERBB2/ERBB3 heterodimer, and ERBB4 homodimer. The dimerization of the receptors activates downstream signaling pathways such as PI3K/AKT, MAPK, and JAK/STAT pathways. Abbreviations: AR, amphiregulin; BTC, betacellulin; EGF, epidermal growth factor; EPG, epigen; EPR, epiregulin; ERK, extracellular signal-regulated kinase; HB-EGF, heparin-binding EGF-like growth factor; JAK, janus kinase; MEK, mitogen-activated protein kinase kinase; mTOR, mammalian target of rapamycin; NRG, neuregulin; PI3K, phosphoinositide 3-kinase; STAT, signal transducer and activator of transcription; TGFα, transforming growth factor-α. Based on (Black, Longo and Carroll, 2019).

III (leucine-rich repeat domains) bind a ligand, and domains II and IV (cysteine-rich

domains) form the tethered conformation with autoinhibitory interactions between these domains (Burgess *et al.*, 2003). Of the four receptors, EGFR, ERBB3, and ERBB4 are in a tethered conformation when they are not binding a ligand (Figure 1A). This conformation prevents dimerization because the dimerization arm in domain II is buried in domain IV, stabilizing the tethered conformation (Burgess *et al.*, 2003).

2.4.4 Unique structural elements in ERBB receptors

ERBB2 is an orphan receptor that does not have a known ligand. However, ERBB2 is constantly in an open conformation, resembling the ligand-bound ERBB receptor (Figure 1A) (Thor *et al.*, 2000; Garrett *et al.*, 2003). The dimerization arm is exposed on the receptor surface, and the ERBB2 receptor is able to form homodimers without a ligand if overexpressed on the cell surface (Ghosh *et al.*, 2011). ERBB2 is also the preferred binding partner to other ERBB family members (Tzahar *et al.*, 1996; Graus-Porta *et al.*, 1997).

ERBB3 is a unique protein of the ERBB protein family as it is a pseudokinase. ERBB3 has no to little kinase activity due to point mutations in its kinase domain (Jura, Shan, *et al.*, 2009). Additionally, ERBB3 is the only ERBB receptor unable to activate signaling through homodimerization (Stein and Staros, 2000). However, ERBB3 is still able to heterodimerize but can function only as an activator kinase.

ERBB4 has also unique characteristics. These include its four different splicing isoforms and their various roles in ERBB4 signaling. The four different isoforms contain two different extracellular juxtamembrane versions (JM-a and JM-b) and two different versions of the carboxyterminal tail (CYT-1 and CYT-2). Unlike the JM-a isoform, the JM-b isoform does not contain the TNFα-converting enzyme (TACE/ADAM17) cleavage site and cannot yield the ERBB4 intracellular domain fragment that has specific roles in intracellular trafficking (Rio *et al.*, 2000; Segers *et al.*, 2020). The CYT-2 isoform does not contain a short sequence of residues from 1046–1061, which the CYT-1 isoform does contain. This part of the sequence includes a phosphorylation site Y1056 which binds p85, a regulatory subunit mediating the phosphatidylinositol 3-kinase (PI3K)/AKT pathway activation (Cohen *et al.*, 1996).

2.4.5 ERBB receptor activation

ERBB receptors can form either homo- or heterodimers, depending on the available receptors and ligands (Figure 1B). The binding of a ligand to a receptor breaks the tethered conformation and causes a conformational change exposing the dimerization arm in domain II. This allows the dimerization arm to contact another

ERBB molecule and for the extracellular domains of ERBB receptors to dimerize. This involves movement at the junction with the transmembrane segments where the C-terminal ends are brought close together (Burgess *et al.*, 2003). These transmembrane segments are connected to the cytoplasmic JM regions of the receptor.

The JM region in ERBB receptors potentiates kinase activity and is involved in the dimerization of the kinase domains (Figure 2) (Thiel and Carpenter, 2007; Jura, Endres, *et al.*, 2009). The JM region of the ERBB receptor consists of JM-A (N-terminal half) and JM-B (C-terminal half) (Jura, Endres, *et al.*, 2009). A clamp is formed by the JM-B portion of the JM region. In an asymmetric dimer, this clamp moves across from the N-terminal lobe of the receiver kinase domain (Jura, Endres, *et al.*, 2009). This is followed by the clamp engaging the C-terminal lobe of the activator kinase domain. Dimerization and the following activation require both the receiver's and the activator's JM-A regions (Jura, Endres, *et al.*, 2009). These two JM-A regions form short α-helices in an asymmetric kinase domain dimer that interact with each other and form a JM-A helical dimer (Jura, Endres, *et al.*, 2009). This helical dimer is stabilized by ligand binding, which further stabilizes the asymmetric kinase domain dimer (Figure 2).

The ERBB receptor's TKD consists of a large carboxy-terminal lobe (C-lobe, activator) and a small amino-terminal lobe (N-lobe, receiver). The N-lobe includes a five-stranded antiparallel β-sheet (Taylor *et al.*, 2012), a regulatory αC-helix, and a conserved glycine-rich ATP-phosphate-binding loop (P-loop). In the formation of the active state (αC-in conformation), a salt bridge is observed between the β3-lysine (K745 in EGFR) and the αC-glutamate (E762 in EGFR). When the receptor is inactive, the two amino acids do not make contact. The C-lobe contains the catalytic loop, the activation loop (containing DFG), the αF-helix, and an activation segment. The catalytic properties of the ERBB receptor come from four amino acids: K/E/D/D

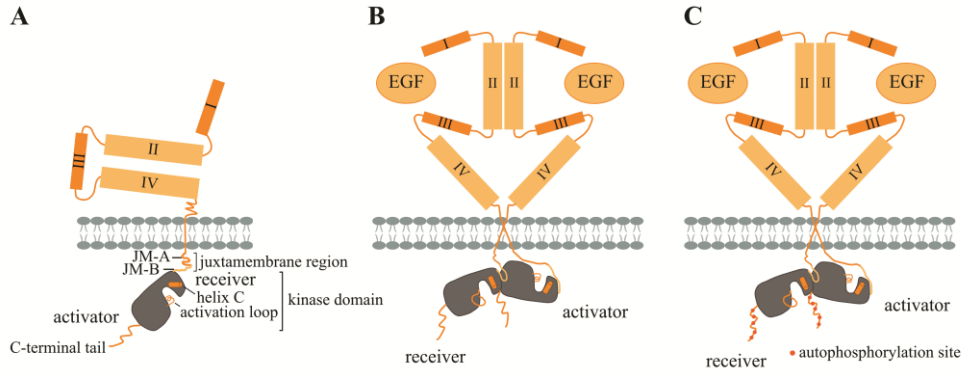

Figure 2. Activation of EGFR homodimer. **A,** Structural elements of an inactive receptor. **B,** EGF-bound receptors form an asymmetric dimer. **C,** Active receptor homodimer with autophosphorylated C-terminal tails. Based on (Jura, Endres, *et al.*, 2009).

(K745, E762, D837, and D855 in EGFR) (Hanks, Quinn and Hunter, 1988), which are conserved among EGFR, ERBB2, and ERBB4. The catalytic loop includes amino acids HRDLAARN in EGFR/ERBB2/ERBB4. ERBB3 has an N instead of D (HRNLAARN), a histidine in place of αC-glutamate and a shortened αC-helix compared to EGFR/ERBB2/ERBB4 structures, thus making ERBB3 catalytically impaired (Roskoski, 2014). The A-loop contains about 20 amino acids in ERBB kinases and one phosphorylatable tyrosine (Y869 in EGFR, Y877 in ERBB2, and Y875 in ERBB4) (Zhang *et al.*, 2006; Qiu *et al.*, 2008).

In addition to conformational changes in the juxtamembrane region, the ERBB receptor TKDs are also subjects to conformational changes and form an asymmetric dimer when activated. The C-lobe contacts the N-lobe of another TKD, inducing conformational changes in the N-lobe of the receiver kinase leading to disruption of *cis*-autoinhibitory interaction. The receiver kinase can then adopt the active configuration. This does not require phosphorylation of the activation loop (Ogiso *et al.*, 2002; Lemmon and Schlessinger, 2010). The phosphorylation of tyrosine residues of the activator kinase is catalyzed by the activated receiver kinase. Oncogenic mutations can result in the disruption of *cis*-autoinhibitory interactions without ligand binding (Sharma *et al.*, 2007).

When EGF binds to EGFR and forms a heterodimer with ERBB2, the EGFR receptor binding the ligand adopts the receiver position in the asymmetric dimer (Zhang *et al.*, 2006). The other receptor (ERBB2) functions as an activator kinase. However, in the case of EGFR, ERBB2, or ERBB4 homodimers, the receptors can adopt either receiver or activator function. In the case of ERBB2/ERBB3 heterodimer formation, ERBB3 can only function as an activator and ERBB2 as a receiver because of the ERBB3's catalytic imparity (Black, Longo and Carroll, 2019). As the catalytic activity of the activator kinase is not required in the allosteric activation mechanism of ERBB receptors, ERBB3 can thus form active heterodimers with other members of the protein family (Guy *et al.*, 1994; Zhang *et al.*, 2006; Shi *et al.*, 2010).

2.4.6 Downstream proteins and signaling

ERBB receptor activation leads to several downstream signaling pathway activations which participate in different cellular processes (Seshacharyulu *et al.*, 2012). The activation of the ERBB receptor's TKD and the resulting autophosphorylation of several C-terminal tail tyrosine residues provide docking sites for adaptor proteins that include Src-homology 2 (SH2) or phosphotyrosine-binding (PTB) domains (Shoelson, 1997; Sudol, 1998; Blume-Jensen and Hunter, 2001). The binding of these proteins further activates subsequent signaling pathways (Olayioye *et al.*, 2000; Yarden and Sliwkowski, 2001; Yu and Jove, 2004).

2.4.6.1 MAPK signaling pathway

One of the main downstream signaling pathways that all ERBB receptors activate is the mitogen-activated protein kinase (MAPK/ERK) pathway. Growth factor receptor bound protein 2 (GRB2) is a docking protein that functions as an adaptor protein mediating the signaling pathway activation. GRB2 is upstream from RAS and binds to several phosphotyrosine residues in all the ERBB receptors (Wilson *et al.*, 2009). Another adaptor protein of the signaling pathway is SHC which also has binding sites in all the ERBB family receptors. Upon binding to EGFR, SHC becomes phosphorylated at Y317 and a binding site for GRB2 (Pelicci *et al.*, 1992; Salcini *et al.*, 1994). The activation of the RAS/RAF/MEK/ERK1/2 signaling pathway is thus mediated by the binding of GRB2 or SHC to the specific phosphotyrosines (Figure 3) (Roskoski, 2010, 2012b). The exchange of GTP to GDP is mediated by son of sevenless (SOS), which is a RAS-guanine nucleotide exchange factor. This exchange of a nucleotide allows RAS to directly interact with its target effectors such as RAF. RAF functions as the initiating kinase of the ERK1/2 module. GRB2 binds to SOS through a SH3 domain in GRB2. When GDP is removed from RAS by SOS, RAS becomes active and induces the protein kinase activity of RAF kinase. RAF subsequently phosphorylates and activates MEK. MEK then phosphorylates and activates ERK1/2. ERK1/2 substrates include protein kinases and transcription factors that lead to cell division (Roskoski, 2012a).

2.4.6.2 PI3K/AKT signaling pathway

PI3Ks form a family of lipid kinases that function by phosphorylating the 3'-OH group of the inositol ring in inositol phospholipids. PI3Ks in class I are heterodimers that contain a catalytic subunit of p110 and an adaptor/regulatory subunit of p85, along with two SH2 domains (Blume-Jensen and Hunter, 2001). ERBB3 is the most important activator of the PI3K/AKT signaling pathway out of ERBB protein family receptors with six phosphotyrosines that can bind the regulatory subunit (p85) of PI3K (Figure 3). ERBB4 can also activate the PI3K pathway directly, but EGFR and ERBB2 activate PI3K/AKT signaling only indirectly through GRB2-associated binding protein 1 (GAB1) (Soltoff and Cantley, 1996). The phosphorylation of membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP2) to form phosphatidylinositol 3,4,5-trisphosphate (PIP3) is catalyzed by p110. The hydrolysis of PIP3 to form PIP2 and inorganic phosphate is catalyzed by PTEN, which functions as a negative regulator of the pathway. AKT is a protein-serine/threonine kinase that binds PIP3 with high affinity (Engelman, 2009; Vanhaesebroeck, Stephens and Hawkins, 2012). AKT can also be activated through the binding of phosphoinositide-dependent protein kinase 1 (PDK1). The phosphorylation of AKT Thr308 is catalyzed by PDK1, and the phosphorylation of AKT Ser472 is catalyzed

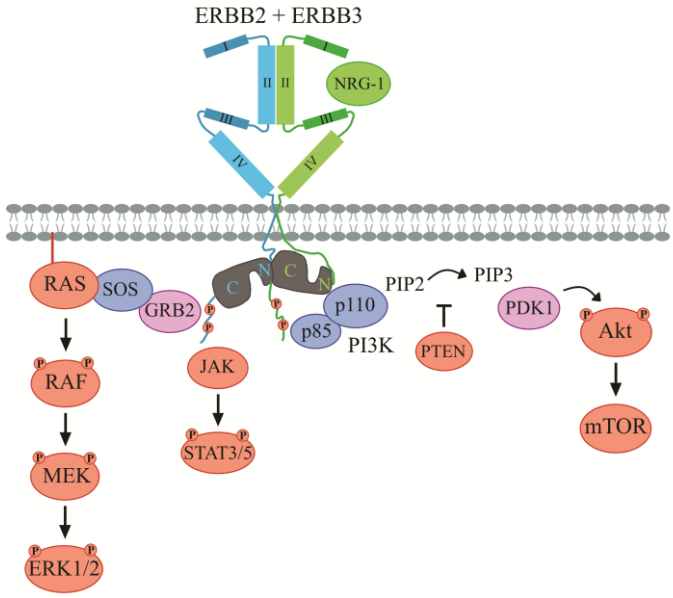

Figure 3. Downstream pathway activation. The dimerization of the receptors can activate different downstream signaling pathways. Abbreviations: GRB2, growth factor receptor bound protein 2; ERK, extracellular signal-regulated kinase; JAK, janus kinase; MEK, mitogen-activated protein kinase kinase; mTOR, mammalian target of rapamycin; NRG, neuregulin; P, phosphorylation; PDK1, phosphoinositide-dependent protein kinase 1; PI3K, phosphoinositide 3-kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PTEN, phosphatase and tensin homolog; SOS, son of sevenless; STAT, signal transducer and activator of transcription.

by the mammalian target of rapamycin complex 2 (mTORC2). The bisphosphorylated and activated AKT then catalyzes the phosphorylation and activation of the mammalian target of rapamycin (mTOR) which is a protein-serine/threonine kinase like AKT (Engelman, 2009). mTOR has several substrates, and it is involved in several cellular processes.

2.4.6.3 JAK/STAT signaling pathway

Janus kinases (JAKs) are essential mediators of cellular signaling through cytokine receptors and belong to the mammalian nonreceptor tyrosine kinase families (Aringer *et al.*, 1999). There are four members of the JAK family: JAK1, JAK2, JAK3, and TYK2. Signal transducer and activator of transcription (STAT) proteins contain seven members: STAT1-4, STAT6, STAT5a, and STAT5b proteins (Darnell, 1997; Yu and Jove, 2004). STAT proteins can function as cytoplasmic signaling proteins or as nuclear transcription factors. The activation of the STAT signaling pathway may function through JAK activation (JAK-dependent) or bypass

JAK activation (JAK-independent) by interacting with STAT proteins directly (Bowman *et al.*, 2000). The activation of the STAT signaling pathway through JAK is initiated by ligand-mediated receptor activation, which leads to induced autophosphorylation of the receptor-associated JAKs and to the transphosphorylation of tyrosine residues within the receptor's cytoplasmic domain (Figure 3). These phosphorylated receptor sites further serve as docking sites that recruit inactive cytoplasmic STAT monomers.

EGFR and ERBB4 can activate the STAT signaling pathway without the involvement of JAK (Xia *et al.*, 2002; Clark *et al.*, 2005). Activated kinases, with their cytoplasmic tails phosphorylated, provide docking sites for the SH2 domains of inactive cytoplasmic STAT monomers, and these are recruited to the activated receptors (Levy and Darnell, 2002). STAT monomers are phosphorylated and form activated dimers through reciprocal phosphotyrosine-SH2 interactions between monomers (Darnell, 1997). This is followed by a STAT dimer moving to the nucleus, where it regulates gene transcription. EGFR can mediate the activation of STAT1, -3, and -5. STAT5 can be activated by EGFR through the Rous sarcoma virus protein tyrosine kinase (SRC). The intracellular domain of ERBB4 can interact with STAT5a (Williams *et al.*, 2004).

2.4.7 ERBB internalization and downregulation

ERBB receptor downregulation can be mediated by several factors that include reversible and irreversible mechanisms. Rapid downregulation mechanisms include endocytosis-mediated receptor degradation and receptor dephosphorylation. A delayed negative regulatory mechanism involves protein inhibitors (Citri and Yarden, 2006). Protein tyrosine phosphatase (PTP) enzymes catalyze the removal of phosphate groups from phosphotyrosines and lead to the inactivation of RTKs (Tonks, 2006). PTPs that specifically target ERBB receptors include PTP1B, PTPN6, and PTPN13 (Liu and Chernoff, 1997; Keilhack *et al.*, 1998; Haj *et al.*, 2003; Zhu *et al.*, 2008).

The major mechanism for ERBB downregulation is receptor endocytosis. The ERBB receptors are internalized from the plasma membrane constitutively, and this internalization rate is increased by ligand binding. The receptors can be recycled back to the plasma membrane (through recycling endosomes) or be trafficked to multivesicular bodies, which are then sorted for lysosomal degradation through ubiquitination.

Ligand-inducible protein inhibitors form another mechanism for negative feedback loop and downregulation. The negative regulators of signaling include a suppressor of cytokine signaling-5 (SOCS-5), which associates with EGFR and drives the suppression of its mitogenic activity, promoting receptor degradation

(Nicholson *et al.*, 2005). MIG-6/RALT binds directly to the tyrosine kinase domain αI-helix of EGFR and allosterically inhibits its catalytic activity (Rubin, Gur and Yarden, 2005; Anastasi *et al.*, 2007).

2.4.7.1 EGFR ubiquitination and endocytosis

EGFR can be internalized by endocytosis via clathrin-coated pits (Carpentier *et al.*, 1982; Hanover, Willingham and Pastan, 1984; Stang *et al.*, 2004; Johannessen *et al.*, 2006) or through endosomes (Honegger *et al.*, 1987; Wiley *et al.*, 1991). The degradation of unoccupied receptors is slow, but the binding of a ligand results in the dramatic acceleration of internalization (Wiley *et al.*, 1991). EGFR binds CBL when tyrosine phosphorylated (Levkowitz *et al.*, 1998). Tyrosine 1069 of EGFR functions as a binding site for the CBL (Levkowitz *et al.*, 1999). The binding of CBL leads to ubiquitinylation of EGFR and to lysosomal degradation of the receptor (Levkowitz *et al.*, 1998). EGFR downregulation appears to need the binding of GRB2 (Wang and Moran, 1996). GRB2 mediates the binding of CBL to EGFR, and studies have shown that this complex alone can be sufficient for receptor endocytosis (Huang and Sorkin, 2005).

CIN85 and endophilin complex formation can also promote receptor endocytosis into early endosomes (Soubeyran et al., 2002). Moreover, EPS15 interacts with the clathrin adaptor protein AP-2 on receptor activation, and this complex can bind ubiquitinated EGFR through ubiquitin-interacting motifs (UIMs), thus promoting receptor endocytosis. Another E3 ubiquitin ligase, Parkin, can limit receptor endocytosis by functioning through PI3K/AKT pathway and by binding the UIM of EPS15 (Fallon et al., 2006).

2.4.7.2 Ubiquitination and endocytosis of other ERBB receptors

EGFR is not the only ERBB receptor that can be ubiquitinated. ERBB2, ERBB3, and ERBB4 also get ubiquitinated, but this does not involve interactions with CBL. ERBB2 ubiquitination involves the carboxyl-terminal HSP70-interacting protein (CHIP), which is an E3 ligase that contains a RING-related U-box (Zhou *et al.*, 2003; Deshaies and Joazeiro, 2009; Xu *et al.*, 2009) or Cullin5 which can interact with both HSP70 and HSP90 (Ehrlich *et al.*, 2009). RING-type E3 ligase neuregulin receptor degradation protein-1 (NRDP1) and a neural precursor cell expressed developmentally downregulated protein 4 (NEDD4) are responsible for ERBB3 ubiquitination (Diamonti *et al.*, 2002; Qiu and Goldberg, 2002; Huang *et al.*, 2015). The steady-state levels of ERBB3 and ERBB4 are regulated by NRDP1 (Diamonti *et al.*, 2002). Soluble ERBB4 ICD is degraded in proteasomes by a multisubunit

RING-type E3 ligase anaphase-promoting complex/cyclosome (APC/C) (Strunk *et al.*, 2007).

ERBB2 is considered to be resistant to downregulation (Baulida *et al.*, 1996; Hommelgaard, Lerdrup and Van Deurs, 2004). This may be due to inefficient internalization or efficient recycling of endocytosed ERBB2 back to the plasma membrane (Roepstorff *et al.*, 2008). ERBB3 is not endocytosed through ligand binding. It is processed by slow endocytosis and with ligand degradation that is reported to take place quite late. This is then followed by rapid recycling (Waterman *et al.*, 1998, 1999). Heterodimers of ERBB2/ERBB3 also undergo slow endocytosis. ERBB4 endocytosis depends on the isoform: CYT-1 isoforms can be endocytosed, while CYT-2 isoforms cannot (Sundvall *et al.*, 2008). Ubiquitination of ERBB4 CYT-1 is catalyzed by Itch (Sundvall *et al.*, 2008).

2.5 ERBB receptors in cancer

Bernard Weinstein introduced the term "oncogene addiction" in 2000, meaning that tumor cell survival depends on the pathway that is activated for its growth (Weinstein, 2000, 2002). ERBB receptor alterations are an excellent example of oncogene addiction (Weinstein, 2000, 2002; Torti and Trusolino, 2011). ERBB receptors have been linked to the development of a number of cancer types by maintaining constitutive signaling due to genomic alterations. The most common types of genomic alterations found within ERBB receptor family members include gene amplifications and point mutations, generally found within specific cancer types. The type of genomic alteration affects diagnostics and what drugs can be used. Oncogene addiction has made ERBB receptors excellent targets for selective drugs. Thus, several antibodies and tyrosine kinase inhibitors (TKIs) have been developed for clinical use to target one or several ERBB receptors in various types of cancer (Figure 4).

2.5.1 EGFR in cancer

EGFR is a significant oncogene and genetically altered in several types of cancer. EGFR is found to be amplified in malignancies such as glioblastoma, lung, colorectal, head and neck squamous cell carcinoma (HNSCC), and esophageal squamous cell carcinoma (Ohgaki *et al.*, 2004; Sunpaweravong *et al.*, 2005). EGFR amplification is also often accompanied by structural rearrangements in gliomas, leading to in-frame deletions in the ECD of the receptor.

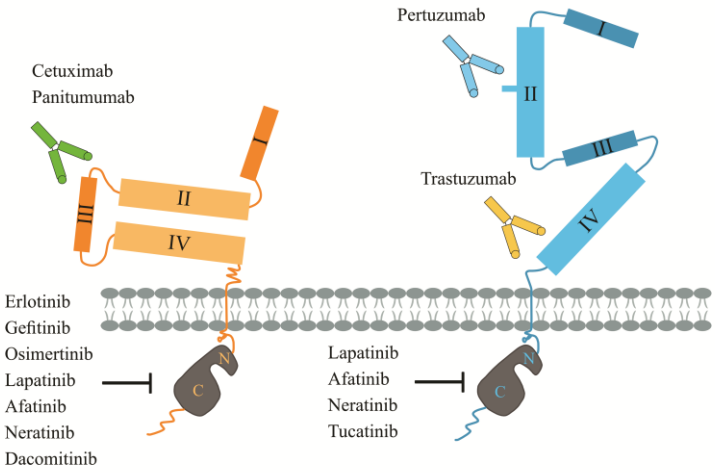

Figure 4. ERBB-targeting compounds, including antibodies and kinase inhibitors. EGFR in orange and ERBB2 in blue.

2.5.1.1 Activating mutations in *EGFR*

EGFR is mutated in several types of cancer, such as NSCLC, and these mutations are distributed over specific regions (Figure 5). 10–40% of lung cancers contain mutations in the EGFR kinase domain (Herbst, Heymach and Lippman, 2008), ranging from 10% in the Caucasian population to 30–40% in the Asian population. The most common mutations identified in EGFR (90% of the observed mutations in NSCLC) are exon19 deletion (residues 746–750) and L858R. The EGFR exon19 deletion has a few variations near the αC-helix (in the N-lobe), and the L858R mutation is located within the hydrophobic core (Shih, Telesco and Radhakrishnan, 2011). Exon19 deletion at the start of the αC-helix (residues 747–753) alters the hydrophobic and hydrophilic interaction networks. Exon19 deletion mutant version of the receptor alters the conformation of the αC-helix by shortening the connecting subdomain. This structural alteration shifts the conformation towards the active conformation (monomeric state). Under EGF stimulation and once the asymmetric dimer forms, the active conformation is destabilized (Choi, Mendrola and Lemmon, 2007). L858R increases the coupling of the A-loop and C-loop (hydrogen bond between G857-H835) and the A-loop to the C-lobe (interactions between R858-R889). The change from a hydrophilic L to a hydrophilic R, particularly in the R-spine and the hydrophobic core, disrupts the hydrophobic interaction networks and shifts them into the perturbation-sensitive region (Shih, Telesco and Radhakrishnan, 2011).

The most frequent mutant variant in glioblastoma is EGFRvIII (Libermann *et al.*, 1985; Sugawa *et al.*, 1990; Ekstrand *et al.*, 1992). EGFRvIII is an oncogenic

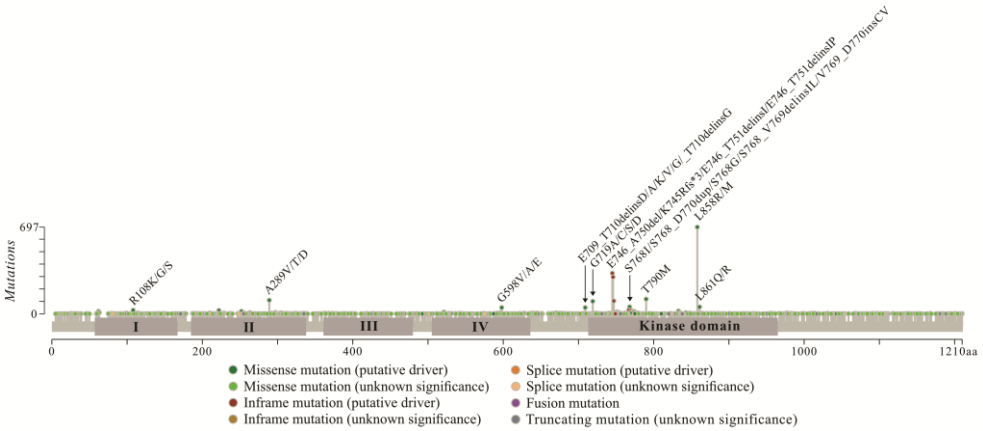

Figure 5. EGFR mutations found in patient samples. Missense, nonsense, indel, truncating, splice, or fusion mutation types. Data derived from cBioPortal (accessed in November 2022).

mutation that deletes exons 2–7 in the receptor ectodomain (residues 6–273). About 40% of high-grade gliomas express EGFRvIII, and are also concurrently found with wild-type EGFR amplification (Sugawa *et al.*, 1990). The EGFRvIII variant has also been identified in a subset of breast, lung, head and neck, prostate, and ovarian cancers (Moscatello *et al.*, 1995). EGFRvIII functions by exhibiting a constitutively active tyrosine kinase and constitutive dimerization, although it is not able to bind a ligand. EGFRvIII demonstrates also impaired downregulation (Nishikawa *et al.*, 1994).

EGFRc958 is the second most common EGFR variant in GBM (identified in about 20% of tumors). EGFRc958 lacks amino acids 521–603, leading to an increased, ligand-dependent kinase activity (Frederick *et al.*, 2000).

About 4–10% of EGFR mutant NSCLC patients harbor EGFR exon 20 insertions (Arcila *et al.*, 2012). The exon 20 insertions comprise a unique set of activating mutations. The most common mutations are located post C-helix and are insertions of one to four residues (Hou *et al.*, 2022). The mutations cause inward movement of the ATP-binding pocket, thus making the TKIs mainly ineffective.

EGFR mutations E709A, G719S, S768I, and L861Q in NSCLS (Lynch *et al.*, 2004; Sordella *et al.*, 2004; Choi, Mendrola and Lemmon, 2007) also function by altering the hydrophilic and hydrophobic interaction networks. The replacement of the hydrophobic L with the hydrophilic Q in L861Q disrupts the hydrophobic core between the A-loop and the αC-helix (Shih, Telesco and Radhakrishnan, 2011). The NSCLC mutation S768I is located at the base of the αC-helix. This mutation affects the hydrophobicity of the site and likely causes a transition of the αC-helix into its active position (Shih, Telesco and Radhakrishnan, 2011). N-lobe mutations E709A and G719S are located near the asymmetric dimer interface. These mutations have been studied to alter the kinase activity by either increasing the dimerization affinity

or by reconfiguring the EGFR RTK monomer (Shih, Telesco and Radhakrishnan, 2011). EGFR mutation A702V was found to be activating in an *in vitro* mutation screen and to likely strengthen the hydrophobic interaction with the activator kinase, which would stabilize the active kinase dimer (Chakroborty *et al.*, 2019).

2.5.1.2 Drugs targeting EGFR

2.5.1.2.1 EGFR antibodies

Cetuximab is an EGFR-neutralizing antibody (a human-mouse chimeric immunoglobulin G1 (IgG1) monoclonal antibody) that has anti-proliferation activities and arrests the target cells in the G1 phase (Peng *et al.*, 1996). Cetuximab blocks ligand binding to EGFR by binding to domain III of the tethered inactive state of EGFR (Figure 4) (Li *et al.*, 2005). Cetuximab synergizes with chemotherapy and has been FDA-approved for the treatment of metastatic colorectal cancer (CRC) that is EGFR wild-type and does not harbor KRAS or NRAS mutations (Cunningham *et al.*, 2004; Allegra *et al.*, 2016). Cetuximab in combination with chemotherapy (Vermorken *et al.*, 2008) or radiotherapy (Bonner *et al.*, 2010) has been approved for the treatment of HNSCC.

Panitumumab is a fully humanized EGFR-targeted monoclonal antibody (IgG2) and has been approved for the treatment of wild-type *RAS* metastatic colorectal cancer (Van Cutsem *et al.*, 2007; Douillard *et al.*, 2013; Kim *et al.*, 2018). Panitumumab binds to the extracellular domain of EGFR with high affinity and blocks the binding of both EGF and TGF (Yang *et al.*, 2001). KRAS mutations are the main source of primary resistance against cetuximab and panitumumab in patients with CRC (Benvenuti *et al.*, 2007; Di Fiore *et al.*, 2007). Mutated KRAS can activate downstream signaling independent of EGFR activation and suppress apoptosis (Downward, 1998; Misale *et al.*, 2012; Zhou, Ji and Li, 2021).

2.5.1.2.2 EGFR TKIs

Gefitinib and erlotinib are first-generation TKIs targeting the wild-type and mutated form of EGFR in NSCLC. They are low molecular weight TKI compounds inhibiting the enzymatic function of EGFR in tumors by competing with ATP. The mutant forms of EGFR have a higher affinity for these first-generation EGFR inhibitors when compared to the wild-type receptor (Carey *et al.*, 2006; Yun *et al.*, 2007). This allows for a therapeutic window for the use of the drugs in NSCLC treatment. Recurrent adverse effects include rash and diarrhea. The generation of a secondary mutation, a gatekeeper mutation T790M, makes the patients resistant to these first-

generation TKIs. Thus, drugs have been developed to specifically target the T790M mutant form of the receptor.

The second-generation TKIs include afatinib, neratinib, and dacomitinib. These three drugs are pan-ERBB inhibitors that irreversibly inhibit all kinase-competent ERBB receptors (EGFR/ERBB2/ERBB4) (Li *et al.*, 2008; Zhu *et al.*, 2015). These drugs are irreversible ATP competitors that form covalent links with the C797 residue of EGFR. These pan-ERBB inhibitors demonstrate activity not only on the sensitizing EGFR mutants, but also on the T790M resistance mutant. However, while these drugs inhibit T790M, they also inhibit wild-type EGFR and create toxicities such as diarrhea and rash. Afatinib has been FDA-approved for metastatic EGFR mutation-positive NSCLC. Dacomitinib has been FDA-approved for the first-line treatment of patients with EGFR-mutated metastatic NSCLC (Wu *et al.*, 2017).

A third-generation TKI osimertinib is a highly active compound in NSCLC patients harboring the resistance mutation T790M acquired after erlotinib or gefitinib treatment (Mok *et al.*, 2017). Osimertinib has been FDA-approved for metastatic EGFR T790M mutation-positive NSCLC, as a first-line treatment for EGFR-mutated metastatic NSCLC and as an adjuvant treatment of patients with early-stage EGFR-mutated NSCLC (Jänne *et al.*, 2015; Goss *et al.*, 2016; Mok *et al.*, 2017; Soria *et al.*, 2018; Herbst *et al.*, 2020; Ramalingam *et al.*, 2020). However, resistance to osimertinib can be acquired through different mechanisms, such as a tertiary *EGFR* mutation C797S (Leonetti *et al.*, 2019).

2.5.2 ERBB2 in cancer

ERBB2 (also known as HER2) is another significant oncogene among ERBB receptors. ERBB2 amplification has been linked to several types of cancer and is targetable by ERBB2 antibodies. ERBB2 is well known to be amplified in about 15–20% of breast cancers (Slamon *et al.*, 1987). ERBB2 is also amplified in a subset of gastric, ovarian, bladder, small bowel, lung, and salivary cancers (Hynes and Stern, 1994; Ménard *et al.*, 2001; Holbro and Hynes, 2004; Hirsch, Varella-Garcia and Cappuzzo, 2009; Kim *et al.*, 2011).

2.5.2.1 Activating mutations in *ERBB2*

ERBB2 mutations have also been identified in various frequencies in different cancer types (Figure 6). ERBB2 mutations are most commonly found in breast, lung, bladder, colorectal, and gastric cancer samples. Activation of ERBB2 can result from different types of mutations: missense mutations in the kinase domain, missense mutations in the extracellular domain, small insertions, or large deletions in the extracellular domain that results in a truncated form of ERBB2 (Arcila *et al.*, 2012;

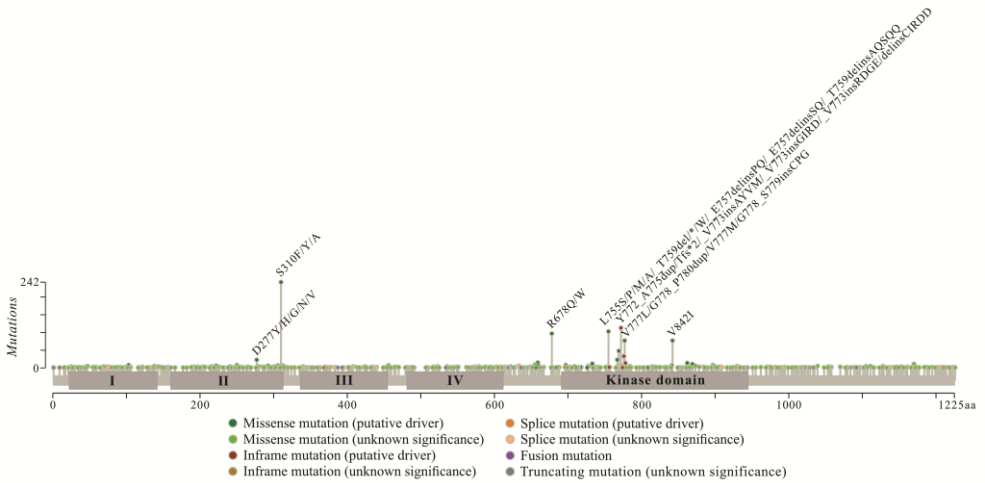

Figure 6. ERBB2 mutations found in patient samples. Missense, nonsense, indel, truncating, splice, or fusion mutation types. Data derived from cBioPortal (accessed in November 2022).

Bose *et al.*, 2013). Unlike EGFR mutations, ERBB2 mutations are generally mutually exclusive with ERBB2 gene amplification (Ross *et al.*, 2016).

p95HER2 lacks substantial parts of the ECD and is thus a truncated form of the protein (Arribas *et al.*, 2011). It is found mainly in breast cancers and may cause resistance to trastuzumab (Molina *et al.*, 2002; Scaltriti *et al.*, 2007).

ERBB2 mutations can also be found in a subset of lung adenocarcinomas (Arcila *et al.*, 2012). These mutations include small insertions in exon 20 (3, 6, 9, or 12 nucleotides between codons M774 and A775). These mutations are mutually exclusive with EGFR, KRAS, and ALK alterations (Arcila *et al.*, 2012). A 12-base pair insertion in exon 20 is the most common insertion mutation in ERBB2. This area of ERBB2 is uniquely hydrophobic in the ERBB family. The insertion mutations weaken the hydrophobic interactions surrounding the αC-β4 loop and form a hydrophilic interaction network similar to those observed with EGFR (Shih, Telesco and Radhakrishnan, 2011).

ERBB2 activating mutation del.755-759 is equivalent to the EGFR exon 19 deletion (Lynch *et al.*, 2004; Paez *et al.*, 2004; Pao *et al.*, 2004). The V777L mutation is homologous to EGFR V769L and ALK F1174L mutations (Chen *et al.*, 2008; George *et al.*, 2008). The EGFR V769L is a rare NSCLC mutation, but it is suggested that the EGFR V769L and ERBB2 V777L mutations would affect tyrosine kinase activity based on their location in the αC-helix (Jura *et al.*, 2011; Bose *et al.*, 2013).

The ERBB2 L755S was originally identified from an *in vitro* mutagenesis screen and is known for its resistance to lapatinib (Trowe *et al.*, 2008; Kancha *et al.*, 2011). The binding site to the small-molecule kinase inhibitors is in close proximity to the ERBB2 L755 side chain. The kinase domain mutations V842I and R896C are located in the C-lobe (Bose *et al.*, 2013). The ERBB2 kinase mutants D769H and V842I

have shown greater tyrosine kinase-specific activity when compared to the wild-type and increases in dimer formation *in vitro* (Bose *et al.*, 2013). The L869R mutation is found in the activation loop of the ERBB2 kinase domain. This site is homologous to BRAF V600E (Wong and Ribas, 2016) and EGFR L862R/Q (Kobayashi and Mitsudomi, 2016).

The ERBB2 ECD mutations G309E, S310F, and S310Y have also been shown to be activating. The G309 and S310 contact the dimerization arm of EGFR in EGFR/ERBB2 heterodimer (Bose *et al.*, 2013; Diwanji *et al.*, 2021). The G309E mutation has been shown to promote covalent homodimerization that is mediated by intermolecular disulfide bond formation (Greulich *et al.*, 2012). The S310F mutation has been shown to function more similarly to the kinase domain mutations and to increase C-terminal tail phosphorylation (Greulich *et al.*, 2012).

2.5.2.2 Treatment options for ERBB2-mutated cancer

2.5.2.2.1 Trastuzumab and pertuzumab

Trastuzumab is an antibody binding to an epitope in the juxtamembrane region IV of the ERBB2 receptor (Figure 4). It is a humanized IgG1 and one of the most important drugs for ERBB2+ breast cancer. Trastuzumab functions by uncoupling ligand-independent ERBB2-containing dimers. This leads to partial inhibition of downstream signaling and to the triggering of antibody-dependent, cell-mediated cytotoxicity (Clynes *et al.*, 2000; Molina *et al.*, 2001; Yakes *et al.*, 2002; Junttila *et al.*, 2009; Ghosh *et al.*, 2011). Trastuzumab also functions by cooperating with the recruitment of a T cell population that mediates an adaptive immune response (Park *et al.*, 2010; Stagg *et al.*, 2011). Trastuzumab has been studied to affect ERBB2 degradation by inducing the internalization and degradation of the ERBB2 receptor (Vu and Claret, 2012). Patients with ERBB2 overexpressing gastric cancer are treated with trastuzumab in combination with cytotoxic therapies (Lee and Ou, 2013). However, trastuzumab has been associated with cardiotoxicity, especially when administered together with anthracyclines (Slamon *et al.*, 2001).

Of the ERBB2-overexpressing metastatic breast cancer patients, only about one-third respond to trastuzumab (single agent) (Cobleigh *et al.*, 1999; Harries and Smith, 2002; Vogel *et al.*, 2002). This intrinsic resistance to trastuzumab can be derived from various reasons, including compensatory pathways and signaling aberrations downstream of ERBB2. Activation of other ERBB receptor homo- or heterodimers through ERBB ligands might partially explain the escape (Lane *et al.*, 2000; Agus *et al.*, 2002; Motoyama, Hynes and Lane, 2002). Trastuzumab binds to a region not involved in receptor dimerization, thus explaining why ERBB ligands can induce the formation of heterodimers containing ERBB2 (Cho *et al.*, 2003).

Pertuzumab is a monoclonal antibody (IgG1) targeting ERBB2. Pertuzumab binds to an epitope in the dimerization domain II of ERBB2 (Figure 4). This monoclonal antibody blocks ERBB2 dimerization (Franklin *et al.*, 2004), the ligand-induced ERBB2/ERBB3 dimerization, and downstream PI3K/AKT signaling (Agus *et al.*, 2002). Pertuzumab is approved by the FDA as a first-line therapy in patients with ERBB2+ metastatic breast cancer, in combination with trastuzumab and docetaxel (Swain *et al.*, 2015).

Trastuzumab and pertuzumab bind to different epitopes in ERBB2, and their combinatorial use has been investigated and shown to have benefits in preclinical and clinical trials (Scheuer *et al.*, 2009; Baselga *et al.*, 2012; Gianni *et al.*, 2012). The combinatorial treatment has been approved for the treatment of ERBB2+ breast cancer patients by the FDA based on clinical trials NeoSphere, APHINITY, and CLEOPATRA (Gianni *et al.*, 2012; Swain *et al.*, 2015; von Minckwitz *et al.*, 2017).

2.5.2.2.2 Antibody-drug conjugates

The ERBB2-targeted antibody-drug conjugate (ADC) ado-trastuzumab-DM1 consists of trastuzumab (stable thioether linker) and a derivative of maytansine (antimitotic agent). This ADC has been approved for use in patients previously treated with trastuzumab and taxane. Prior treatment for metastatic disease or disease recurrence is expected. Ado-trastuzumab emtansine (T-DM1) was FDA-approved in 2013 for metastatic ERBB2+ breast cancer.

Trastuzumab-deruxtecan (DS-82-01a) is another ADC that contains a humanized monoclonal antibody (trastuzumab) and a tetrapeptide linkaged deruxtecan (a topoisomerase 1 inhibitor) (Modi *et al.*, 2020). The drug received FDA accelerated approval in 2019 for patients with unresectable or metastatic ERBB2+ breast cancer (with two or more prior anti-ERBB2 therapies). On August 2022, the drug was approved by FDA for patients with unresectable or metastatic ERBB2 low breast cancer based on DESTINY-Breast04 (NCT03734029) (Modi *et al.*, 2022).

2.5.2.2.3 ERBB2 TKIs

Several TKIs target ERBB2. One of these is lapatinib, which is an ATP-competitive, reversible small-molecule inhibitor that targets both EGFR and ERBB2 (Konecny *et al.*, 2006). Lapatinib is a quinazoline derivative containing side chains that are different from gefitinib, erlotinib, and afatinib. Lapatinib has been studied to disable ERBB2 signaling in ERBB2+ breast cancers and also to inhibit the PI3K/AKT and MAPK pathways. Lapatinib has been shown to have clinical activity in ERBB2+ breast cancer patients that have progressed while on trastuzumab (Geyer *et al.*,

2006). Lapatinib is FDA approved for ERBB2+ advanced or metastatic breast cancer in combination with capecitabine (Ryan *et al.*, 2008).

Neratinib is an irreversible pan-ERBB TKI (EGFR/ERBB2/ERBB4). Neratinib has been studied to decrease phosphorylation of the ERBB receptor's intracellular TKD and to inhibit downstream signaling of PI3K/AKT and RAS/MAPK pathways (Segovia-Mendoza *et al.*, 2015). Neratinib was FDA-approved in 2017 after the results from the ExteNET trial were released. The drug approval was for the extended adjuvant treatment for early-stage ERBB2+ breast cancer patients that have been previously treated with adjuvant trastuzumab (Chan *et al.*, 2016). In 2020, Puma Biotechnology received FDA approval for neratinib to treat ERBB2+ metastatic breast cancer.

Tucatinib is a selective and reversible compound against ERBB2 that binds to and inhibits the intracellular TKD of the ERBB2 receptor. Tucatinib was FDA-approved in 2020 in combination with trastuzumab and capecitabine for advanced unresectable or metastatic ERBB2+ breast cancer patients (Murthy *et al.*, 2018).

2.5.3 ERBB3 in cancer

ERBB3 is another member of the ERBB protein family that is frequently observed in cancer. It is mostly mutated in different cancer types, but overexpression of the receptor is also observed in gastric and ovarian cancers (Tanner *et al.*, 2006; Hayashi *et al.*, 2008). ERBB3 is an important member of the ERBB receptor family due to its role as a significant heterodimerization partner (especially with ERBB2) and as a mediator of drug resistance (Sergina *et al.*, 2007).

2.5.3.1 Activating mutations in *ERBB3*

Somatic mutations of ERBB3 are found particularly in the ECD region of the protein, but some oncogenic mutations are also found in the kinase domain (Figure 7). ERBB3 is often mutated in breast, bladder, colorectal, gastric, and lung cancer (cBioPortal) (Cerami *et al.*, 2012; Gao *et al.*, 2013).

One of the most important ERBB3 mutation studies has been conducted by Jaiswal *et. al.* In this study, whole exome sequencing of *ERBB3* from 100 primary colorectal tumors, along with their matched normal samples, identified mutations in ERBB3 in 11% of the tumors (Jaiswal *et al.*, 2013). The study also investigated 92 gastric cancers and identified mutations in 12% of samples, along with 71 NSCLC adenocarcinomas with a mutation rate of 1%, and 67 squamous cell NSCLCs with a mutation rate of 1%. Recurrently mutated amino acids included V104, A232, P262, G284, D297, G325, and T355 (Jaiswal *et al.*, 2013). From these amino acid sites, the V104, A262, and G284 cluster at the interface between domains I and II. At the base

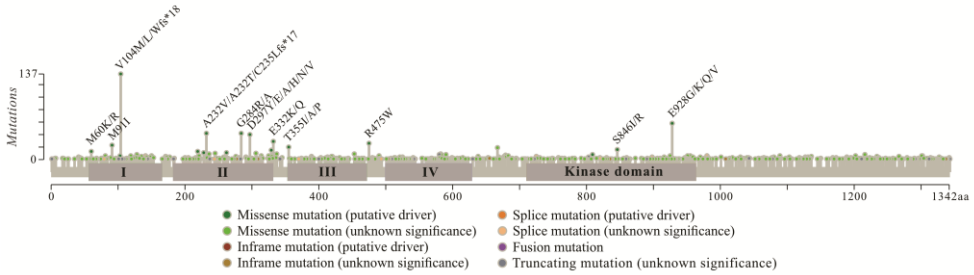

Figure 7. ERBB3 mutations found in patient samples. Missense, nonsense, indel, truncating, splice, or fusion mutation types. Data derived from cBioPortal (accessed in November 2022).

of domain II is located P262. This site is close to Q271, which has been linked to domain II/IV interaction and to the formation of the tethered inactive conformation. D297 is located in domain II and has a role in heterodimerization. Amino acid sites D297, G325, and T355 are located in positions where large conformational transitions happen during the switch from the inactive to active state. T355I mutation would disrupt ERBB3 autoinhibition and make the receptor favor an extended conformation.

Kinase domain mutations S846I and E928G were also found in the study (Jaiswal *et al.*, 2013). Amino acid S846 might be involved in receptor endocytosis, and E928 is near the protein-protein interface that is observed in the asymmetric kinase dimer (Collier *et al.*, 2013; Jaiswal *et al.*, 2013).

2.5.3.2 Treatment options for ERBB3-mutated cancer

There have been efforts in trying to develop a compound that would block ERBB3 signaling, but because ERBB3 is kinase-impaired, the development of a TKI has not been feasible. Thus, efforts have been focusing on ERBB3 antibodies and agents that would block its heterodimerization with other ERBB family members or agents that would prevent ligand binding. There have also been attempts to develop bispecific antibodies targeting ERBB3 and ERBB2. Also, agents promoting ERBB3 destruction by internalization or other means or locking ERBB3 in a tethered conformation have been developed (Xie *et al.*, 2014; Mishra *et al.*, 2018). Several ERBB3-neutralizing antibodies are in clinical development. These include, for example, MM-121 (seribantumab) and U3-1287 (patritumab), which bind the extracellular domain of ERBB3. These antibodies should block the neuregulin-induced phosphorylation and reduce ERBB3 expression at the cell surface (Schoeberl *et al.*, 2010; Garrett *et al.*, 2011). Clinical trials have been investigating the effects of patritumab alone or as an ACD (patritumab deruxtecan, ERBB3-DXd) (LoRusso *et al.*, 2013; Jänne *et al.*, 2022). Patritumab deruxtecan has shown some promise in the clinical setting. Some trials investigating the use of patritumab

deruxtecan are still ongoing (NCT04619004 and NCT05338970). However, quite a few of the clinical studies investigating the use of ERBB3-targeting compounds have been terminated. The failure of most of the drugs might be derived from the conformational and physical plasticity of the ERBB2/ERBB3 heterodimers (Campbell *et al.*, 2022).

2.5.4 ERBB4 in cancer

ERBB4 is also genetically altered in various cancer types. ERBB4 may be mutated at various sites that are distributed across the gene (Figure 8). ERBB4 is mutated in melanomas (Prickett *et al.*, 2009) but also in low numbers in lung (Ding *et al.*, 2008) and other tumors such as CRC (Gilbertson *et al.*, 2001).

ERBB4 has been studied in several cancer types, but its role is still not entirely clear (reviewed in Segers *et al.*, 2020). ERBB4 is a unique member of the ERBB protein family in several ways. One of these ways is its role as an oncoprotein and a tumor suppressor. ERBB4 has been studied to function as a tumor suppressor in cancers such as bladder and cholangiocarcinoma. For example, the elevated expression of ERBB4 in urothelial tumors has been associated with lower-grade, less invasive tumors (Memon *et al.*, 2004, 2006; Black and Dinney, 2008). On the other hand, ERBB4 has been studied to function as an oncoprotein in cancers such as medulloblastoma, CRC, gastric cancer, and HNSCC (Kalyankrishna and Grandis, 2006; Überall *et al.*, 2008; Shi *et al.*, 2012; He *et al.*, 2015; Arienti, Pignatta and Tesei, 2019; Segers *et al.*, 2020). For example, ERBB4 overexpression has been studied to associate with more aggressive CRC tumors and metastatic tendencies (Kountourakis *et al.*, 2006; Baiocchi *et al.*, 2009; Khelwatty *et al.*, 2013; Mitsui *et al.*, 2014; Williams *et al.*, 2015).

2.5.4.1 Activating mutations in *ERBB4*

Activating ERBB4 mutations in NSCLC include Y285C, D595V, D931Y, and K935I. These mutations have been demonstrated to increase ligand-dependent and -independent tyrosine phosphorylation and to increase heterodimerization with ERBB2 (Soung *et al.*, 2006; Ding *et al.*, 2008; Tvorogov *et al.*, 2009; Kurppa *et al.*, 2016).

Several ERBB4 mutations have been identified and characterized in melanoma (Kurppa and Elenius, 2009; Prickett *et al.*, 2009). From these 20 mutations, L39F, R393W, and E872K have also been studied in other cancer types, and E872K to be functionally relevant (Soung *et al.*, 2006; Prickett *et al.*, 2009; Tvorogov *et al.*, 2009). Mutations E317K, E452K, E542K, R544W, E563K, E836K, and E872K have been studied to increase ERBB4 tyrosine kinase activity and phosphorylation.

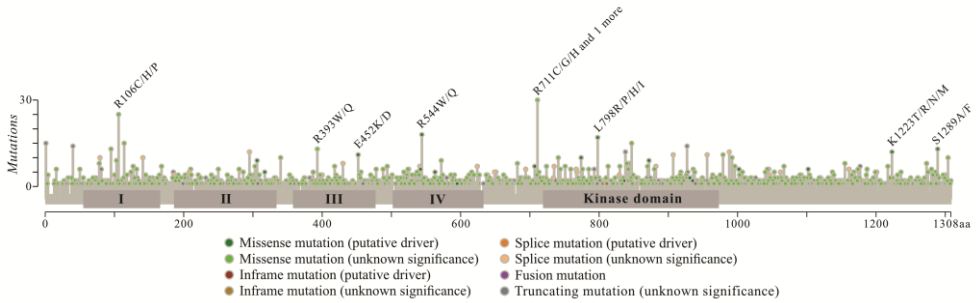

Figure 8. ERBB4 mutations found in patient samples. Missense, nonsense, indel, truncating, splice, or fusion mutation types. Data derived from cBioPortal (accessed in November 2022).

ERBB4 mutations E836K and E872K in melanoma (Prickett *et al.*, 2009) function by altering the hydrophilic and hydrophobic interaction networks as the similar EGFR mutations (Shih, Telesco and Radhakrishnan, 2011). In the paper by Kurppa *et. al.*, ERBB4 mutations Y285C, D595V, D931Y, and K935I were characterized as oncogenic by enhancing both basal and NRG-1-induced ERBB4 phosphorylation and also to increase the activation of endogenous ERBB2 in the presence of NRG-1 (Kurppa *et al.*, 2016). ERBB4 mutations Y285C and D595V efficiently formed ERBB4 homodimers when stimulated with NRG-1 in NIH-3T3 cells. Increased transactivation between ERBB4 monomers was observed with ERBB4 K935I. ERBB4 D931Y and K935I in the C-terminal lobe of the activator kinase demonstrated increased association with ERBB2 when stimulated with a ligand (potent activators of ERBB2/ERBB4 heterodimers). ERBB4 K935I enhanced interaction also between ERBB4 kinase monomers (Kurppa *et al.*, 2016). Other activating ERBB4 mutations include ERBB4 E715K and R687K, which have demonstrated activity in Ba/F3, NIH-3T3, and BEAS-2B cells (Chakroborty *et al.*, 2022). These mutations are found in skin cancer patients (cBioPortal).

2.5.4.2 Treatment options for ERBB4-mutated cancer

There are various pan-ERBB inhibitor compounds and drug responses of cells expressing ERBB4 mutants to ERBB-targeting compounds have been studied. However, there are currently no FDA-approved cancer treatments that specifically target ERBB4. Clinical trials targeting ERBB4-mutated cancer have been conducted, but no clinical responses have been observed (González-Cao *et al.*, 2015; Xu *et al.*, 2018). There is currently one ongoing study (www.clinicaltrials.gov) that is a phase I trial and focuses on the adverse effects and preferred dose of neratinib in combination with everolimus, palbociclib, or trametinib in patients with either EGFR alterations, ERBB2 alterations, ERBB3/ERBB4 mutations, or KRAS mutations (NCT03065387).

2.6 Future directions for personalized medicine

Challenges in personalized cancer medicine include the understanding of the genetics of each cancer, the need for matching the right drug with the individual tumor, the design of rational combinations, the monitoring of the treatment response, and the testing of new anticancer agents earlier in disease (Curigliano, 2012; Wahida *et al.*, 2022).

Because of the high number of bypass mechanisms cancer can develop, there is a need to combine multiple drugs that additionally block the key bypass tracts promoting resistance. However, there are challenges in determining combination treatments. These include the choice of an optimal combination for an individual patient (Arteaga and Engelman, 2014). Thus, there is a great need for the identification of new biomarkers for targeted therapy. Repeated biopsies and next-generation sequencing can give more information about cancer resistance patterns, but there are still limitations in our knowledge of the mechanisms of how the resistance will develop. Taking combination therapies in use at first-line treatment might eliminate the opportunities for resistance clones to arise and adapt. However, the tolerability of the simultaneous use of several compounds can become an issue.

The increasing amount of sequencing data and drug profiling can provide more insight into personalized treatment in the future. The main obstacle is still the heterogeneous nature of a tumor, both inter- and intratumorally. The majority of critical genomic changes occur during cancer progression, which creates significant variability in both primary and metastatic tumors (Cejalvo *et al.*, 2017). Chang *et. al.* also speculated that new mutant alleles are more likely to emerge faster than new cancer genes are identified. This will extend the long tail of the frequency distribution of somatic mutations (Chang *et al.*, 2016). There is already a significant number of rare mutations that remain uncharacterized but might have a functional effect on cancer development and/or be targeted by compounds already available. A major limitation in the identification of these activating, rare mutations is that their identification and validation take a lot of time and resources. Thus, there is a great need for methods that would identify these candidates more efficiently.

Problems with data sharing are limiting discoveries as the data produced by institutes is usually not freely available or in similar formats. Being able to analyze data across multiple institutions increases the number of rare cancer type samples or rare variant samples and increases the statistical power of analysis. Fortunately, precision medicine is moving in a direction where large clinical data and biomarker trials have been initiated and that focus on collaboration and universal data collection and analysis methods (Frank, 2000; Elliott and Peakman, 2008; Vargas and Harris, 2016; Hu and Dignam, 2019). Sufficient funding also from external funders and government agencies, such as the EU's Horizon Europe Mission on Cancer, will accelerate future research.

3 Aims

The general aim of this study was to identify novel activating *ERBB* mutations that could also function as predictive mutations for ERBB-targeted cancer therapeutics. Cancer cells contain thousands of mutations, and only a small part of these mutations are activating mutations responsible for cancer development. The majority of other mutations are passenger mutations with limited function. Although there are several predictive biomarkers in clinical use, there is still a great need for novel predictive mutations that target the treatment toward more personalized medicine.

The frequency distribution of somatically mutated genes across cancer samples has a so-called long tail, meaning that the majority of hotspot mutations, while recurrent, are not common in any specific cancer type (Chang *et al.*, 2016). There are statistical data about this long tail of the mutated genes that imply that there is a significant number of potentially clinically relevant predictive mutations still to be identified (Garraway and Lander, 2013; Chang *et al.*, 2016). Obstacles in identifying potential novel predictive mutations are the rare occurrence at a population level, technical obstacles in the screening of thousands of mutations, the amount of labor and finances the validation process requires, and the lack of structurally defined hotspots. This study introduces two screens, which were able to identify mutations in *ERBB* receptor genes associating with receptor activity and/or sensitivity to ERBB-targeted compounds.

To identify novel predictive *ERBB* mutations, two screens were used. The specific aims for these two screens were as follows:

1) To establish a cancer cell line database screen using three publicly available databases to identify drug-sensitive cell lines harboring an *ERBB* mutation. To identify activating *ERBB* mutations by functionally validating selected *ERBB* mutations and to determine the drug sensitivities of these mutants to ERBB-targeted compounds.
2) To establish an unbiased functional genetics screen to identify activating *ERBB3* mutations from thousands of *ERBB3* mutations in parallel in the context of ERBB3/ERBB2 heterodimers. To functionally validate the transforming and drug-sensitizing properties of the individual growth-activating *ERBB3* mutations.

4 Materials and Methods

4.1 Expression plasmids (I-II)

4.1.1 pDONR-221 constructs (I-II)

Gateway cloning (Addgene; (Chakroborty *et al.*, 2019)) between pDONR-221 and pBABE-gateway constructs was used to generate retroviral expression plasmids. *ERBB* inserts for generating pBABE-puro-gateway-*ERBB3* and pBABE-puro-gateway-*ERBB4JM-aCYT-2* are described in I and (Määttä *et al.*, 2006; Merilahti *et al.*, 2017). The creation of an expression library of random *ERBB3* mutants and wild-type *ERBB3* construct are described in II. Point mutations to *ERBB* inserts in the pDONR-221 background were introduced by site-directed mutagenesis using oligonucleotide primers listed in the supplementary material of I and II. All constructs were verified by Sanger sequencing.

4.1.2 pBABE-gateway constructs (I-II)

Retroviral expression plasmids were used to express the indicated wild-type and mutant proteins in cell lines (4.2). All expression plasmids generated for this study are listed in Table 4. pBABE-neo-gateway-*ERBB2* plasmid was generated by restriction enzymes and ligation as described in II. Previously described expression plasmids are listed in Table 5. pBABE-puro-gateway empty vector (Addgene plasmid No. 51070) and pBABE-puro-gateway-*ERBB2* (Addgene plasmid No. 40978) were gifts from Dr. Matthew Meyerson (Dana Farber Cancer Institute). pBABE-puro-gateway-*EGFR* has been previously described (Chakroborty *et al.*, 2019).

Table 4. Expression plasmids generated in this study.

INSERT	BACKBONE	PURPOSE	USED IN
EGFR G719S	pBABE-puro-gateway	Retroviral	I

EGFR Y1069C	pBABE-puro-gateway	Retroviral	I
ERBB2 E936K	pBABE-puro-gateway	Retroviral	I
ERBB2 L720P	pBABE-puro-gateway	Retroviral	I
ERBB2 S310F	pBABE-puro-gateway	Retroviral	I
ERBB2 V956R	pBABE-neo-gateway	Retroviral	II
ERBB2 wild-type	pBABE-neo-gateway	Retroviral	II
ERBB3 A676T	pBABE-puro-gateway	Retroviral	II
ERBB3 D1259Y	pBABE-puro-gateway	Retroviral	II
ERBB3 D797V	pBABE-puro-gateway	Retroviral	II
ERBB3 E332K	pBABE-puro-gateway	Retroviral	II
ERBB3 E928fs*16	pBABE-puro-gateway	Retroviral	I
ERBB3 E952Q	pBABE-puro-gateway	Retroviral	I
ERBB3 E928G	pBABE-puro-gateway	Retroviral	II
ERBB3 K279N	pBABE-puro-gateway	Retroviral	II
ERBB3 K329R	pBABE-puro-gateway	Retroviral	II
ERBB3 K329R+E332K	pBABE-puro-gateway	Retroviral	II
ERBB3 L361P	pBABE-puro-gateway	Retroviral	II
ERBB3 L482P	pBABE-puro-gateway	Retroviral	II
ERBB3 L361P+L482P	pBABE-puro-gateway	Retroviral	II
ERBB3 N353T	pBABE-puro-gateway	Retroviral	II
ERBB3 P212L	pBABE-puro-gateway	Retroviral	II
ERBB3 wild-type	pBABE-puro-gateway	Retroviral	I, II
ERBB3 Y265C	pBABE-puro-gateway	Retroviral	II
ERBB4 A17V	pBABE-puro-gateway	Retroviral	I
ERBB4 L780P	pBABE-puro-gateway	Retroviral	I
ERBB4 G863E	pBABE-puro-gateway	Retroviral	I
ERBB4 G936R	pBABE-puro-gateway	Retroviral	I

Table 5. Expression plasmids described previously.

INSERT	BACKBONE	PURPOSE	USED IN
EGFR wild-type	pBABE-puro-gateway	Retroviral	I, II
ERBB2 wild-type	pBABE-puro-gateway	Retroviral	I
ERBB4 JM-a CYT-2 wild-type	pBABE-puro-gateway	Retroviral	I

Empty vector	pBABE-puro-gateway	Retroviral	I, II

4.2 Cell culture (I-II)

Cell lines used in this study are listed in Table 6. Phoenix Ampho HEK293T cells (a gift from Dr. Garry Nolan, Stanford University School of Medicine) were used to produce retroviruses used for the stable cell line production (4.2.2). Ba/F3 (DSMZ), NIH-3T3 (ATCC), and MCF-10A (AATC) cells were used for stable expression of proteins from expression plasmids (4.1). COS-7 cells were used for the transient expression of proteins. Ba/F3, MCF-10A, and NIH-3T3 cells were used to study the activating properties of the *ERBB* mutant cell lines (4.6-4.9).

Phoenix Ampho HEK293T cells and Ba/F3 cells were cultured in RPMI-1640 (Lonza) supplemented with 10% FBS (Biowest), ultraglutamine 1 (Lonza), 50 U/ml penicillin and 50 U/ml streptomycin (Gibco). Ba/F3 cell medium was further supplemented with interleukin 3 (IL3) in the form of a 5% conditioned WEHI cell medium when cultured IL3-dependently. COS-7 and NIH-3T3 cells were cultured in DMEM (Lonza) supplemented with 10% FBS, 50 U/ml penicillin, and 50 U/ml streptomycin. MCF-10A cells were cultured in DMEM/F-12 (Lonza) supplemented with 10% FBS, 50 U/ml penicillin, 50 U/ml streptomycin, 20 ng/ml EGF (Peprotech), 0.5 mg/ml hydrocortisone (Sigma), 100 ng/ml cholera toxin (Sigma), and 10 µg/ml insulin (Sigma). Cells were routinely tested for *Mycoplasma* infection using MycoAlert (Lonza).

Table 6. Cell lines used in this study.

CELL LINE	TYPE	SPECIES	USED IN
Ba/F3	Pro B cells	Mouse	I, II
COS-7	Kidney fibroblast-like cells	African green monkey	I
NIH-3T3	Fibroblast cells	Mouse	I, II
MCF-10A	Human breast epithelial cells	Human	II
Phoenix Ampho HEK293T	Embryonic kidney cells	Human	I, II

4.2.1 Transient transfection (I)

For transient transfection of the proteins from expression plasmids (4.1), COS-7 cells were plated on 6-well plates at 100,000 cells/well. The next day, the cells were transfected with the pBABE-puro-gateway-*EGFR*, pBABE-puro-gateway-*ERBB2*, pBABE-puro-gateway-*ERBB3* constructs or their combinations or the empty pBABE-puro-gateway vector using Fugene6 transfection reagent (Promega). A total

of 2 µg of plasmid DNA diluted in Opti-MEM (Gibco) was used per transfection. Cells were used for Western blot analyses 48 hours after transfection (as described in I).

4.2.2 Stable transfection for cell line production (I-II)

pBABE-gateway-based *ERBB* expression vectors were transfected into Phoenix Ampho cells using Fugene6 for the production of infective retroviruses. Ba/F3, MCF-10A, and NIH-3T3 cells were infected with the retroviral supernatants, as previously described in II and (Chakroborty *et al.*, 2019; Koivu *et al.*, 2021). Ba/F3 or MCF-10A cells with integrated retroviral inserts were selected with 2 µg/ml puromycin (Gibco) for 48 hours. NIH-3T3 cells were selected with 6 µg/ml puromycin. Selection pressure was subsequently maintained by the presence of 1 µg/ml or 3 µg/ml puromycin in the culture medium, respectively. To generate Ba/F3, MCF-10A, or NIH-3T3 cells with simultaneous expression of both ERBB2 and ERBB3, stable puromycin-selected cells expressing ERBB3 were transduced with supernatants from Phoenix Ampho cells expressing pBABE-puro-gateway-*ERBB2* (wild-type) (as in I) or pBABE-neo-gateway-*ERBB2* (V956R) (as in II). To select cells expressing pBABE-neo-gateway-*ERBB2,* 500 µg/ml of Geneticin (Gibco) was used for five days, and the selection pressure was subsequently maintained by the presence of 250 µg/ml Geneticin.

4.3 Primary antibodies (I-II)

Primary antibodies (listed in Table 7) were used to detect proteins of interest by Western blotting, in immunoprecipitation (IP) assays, and in cell sorting.

Table 7. Primary antibodies used in this study. Application abbreviations: CS, cell sorting; IP, immunoprecipitation; WB, Western blotting. Company abbreviations: CST, Cell Signaling Technology; SCBT, Santa Cruz Biotechnology; TS, Thermo Scientific.

ANTIGEN	CAT#/CLONE	COMPANY	TYPE	APPLICATION	USED IN
Actin	A5441	Sigma-Aldrich	Mouse monoclonal	WB	I, II
AKT	2920	CST	Mouse monoclonal	WB	I, II
c-CBL	2747	CST	Rabbit polyclonal	IP, WB	I
EGFR	Sc-03	SCBT	Rabbit polyclonal	WB	I
EGFR	4267	CST	Rabbit monoclonal	WB	I
ERBB2	MA-5-14057	TS	Mouse monoclonal	WB	I, II
ERBB2	FAB1129G	R&D Systems	Mouse monoclonal	CS	II

ERBB3	4754	CST	Rabbit monoclonal	WB	I, II
ERBB3	FAB3481R	R&D Systems	Mouse monoclonal	CS	II
ERBB4	E200	Abcam	Rabbit monoclonal	WB	I
ERK	9102	CST	Rabbit polyclonal	WB	I, II
Phospho-AKT	4060	CST	Rabbit monoclonal	WB	I, II
Phospho-EGFR	2220	CST	Rabbit polyclonal	WB	I
Phospho-ERBB2	2243	CST	Rabbit monoclonal	WB	I, II
Phospho-ERBB3	4791	CST	Rabbit monoclonal	WB	I, II
Phospho-ERBB4	4757	CST	Rabbit monoclonal	WB	I
Phospho-ERK	9101	CST	Rabbit polyclonal	WB	I, II
Tubulin	T7816	Sigma-Aldrich	Mouse monoclonal	WB	I

4.4 Growth factors and ERBB targeting drugs (I-II)

Table 8 lists all the growth factors, ERBB tyrosine kinase inhibitors (TKI), and ERBB antibodies used in this study. The concentrations and incubation times in each experiment are indicated in the sections 4.6-4.9.

Table 8. Growth factors and ERBB inhibitors or antibodies used in this study. Company abbreviations: SCBT, Santa Cruz Biotechnology.

REAGENT	APPLICATION	COMPANY	USED IN
EGF	EGFR stimulation	R&D Systems	I
NRG-1	ERBB3/ERBB4 stimulation	R&D Systems	I, II
Afatinib	Inhibition of EGFR/ERBB2/ERBB4	SCBT	I
Erlotinib	Inhibition of EGFR	SCBT	I
Lapatinib	Inhibition of EGFR/ERBB2	SCBT	I
Neratinib	Inhibition of EGFR/ERBB2/ERBB4	SCBT	I, II
Trastuzumab	Inhibition of ERBB2	Genentech	II
Pertuzumab	Inhibition of ERBB2	Genentech	II

4.5 Cell sorting (II)

Sony SH800 Cell Sorter or BD LSRFortessa Cell Analyzer (BD Biosciences) were used to select cells expressing both ERBB2 V956R and ERBB3 wild-type or mutants. Human ERBB2/Her2 Alexa Fluor® 488-conjugated Antibody (FAB1129G, R&D Systems) and Human ERBB3/Her3 Alexa Fluor® 647-conjugated Antibody (FAB3481R, R&D Systems) were used as described in II.

4.6 Ba/F3 cell growth and drug sensitivity analysis (I-II)

The potential of *ERBB* mutations to activate growth was studied by growth analysis in Ba/F3 cell lines with stable expression of the receptors as either homo- or heterodimeric complexes. Ba/F3 cells are optimal to study activating mutations in RTKs as the cell line requires IL3 for growth unless the transduced RTK harbors an activating mutation (Warmuth *et al.*, 2007). Ba/F3 cells were washed twice with PBS and seeded at 100,000 cells/ml in RPMI-1640 supplemented with 10% FBS, with or without IL3 (5% WEHI as a cell conditioned medium), or with or without 10 ng/ml EGF (R&D Systems) or 20 ng/ml NRG-1 (R&D Systems). Quadruplicate aliquots of Ba/F3 cells (as 100 µl aliquots corresponding to 10,000 cells at the initial seeding density) were collected at indicated time points into 96-well plate wells and analysis of cell viability was performed with the MTT assay (CellTiter 96 nonradioactive cell proliferation assay, Promega).

The sensitivity of the ERBB wild-type or mutant cell lines to various ERBB TKIs was studied by drug analysis as described in I-II. Cell growth medium was supplemented as indicated and final concentrations of drugs (Table 8) were as indicated in I-II.

4.7 MCF-10A cell growth (II)

Growth of MCF-10A cells with stable expression of the ERBB3 wild-type or mutants either alone or together with ERBB2 V956R was performed as described in II.

4.8 Focus formation assay (II)

NIH-3T3 cells with stable expression of the ERBB3 wild-type or mutants either alone or together with ERBB2 V956R were grown in DMEM supplemented with 3% FBS for two weeks and fixed, stained, and analyzed as described in II.

4.9 Cell lysis, immunoprecipitation, and western blotting (I-II)

Cell signaling and receptor activity were studied in Ba/F3, NIH-3T3, MCF-10A, and COS-7 cells. NIH-3T3, MCF-10A, or COS-7 cells expressing the indicated receptors were starved overnight in DMEM or DMEM/F-12 in the absence of serum and stimulated for 10 minutes with 0 or 50 ng/ml of ERBB ligands EGF or NRG-1. After ligand stimulation, the cells were washed three times with PBS and suspended in the lysis buffer followed by western blotting analysis as described in (Ojala *et al.*, 2020; I-II). Primary antibodies used to detect proteins are listed in Table 7 (4.3).

Ba/F3 cell samples were collected from confluent, differently conditioned medium conditions. Cells were lysed and analyzed by western blotting, as previously described (Ojala *et al.*, 2020). Primary antibodies used to detect proteins are listed in Table 7 (4.3).

For co-immunoprecipitation of samples, NIH-3T3 cells expressing the proteins were starved overnight in DMEM + 0% FBS. Cells were then stimulated with 0 or 50 ng/ml of EGF for 10 minutes and cells were lysed in a volume of 400 µl. Lysates were prepared for co-immunoprecipitation and Western blot analysis as described in I.

4.10 Obtaining data from cancer cell line databases (I)

For the creation of the *ERBB* mutated cancer cell line drug sensitivity dataset used in I, the data were prepared by searching human cancer cell line, sequencing data, and drug response data as described in I. The relative area under the curve (rAUC) and modeled EC50 (mEC50) values were calculated for each database, cancer cell line, *ERBB* mutation, and ERBB compound combination as described in I. Potential predictive mutations were identified from the dataset by selecting cell lines with a rAUC value of ≥ 1.0 and a mEC50 value of ≤ 1.0 µM for a given drug, as described in I.

4.11 Next-generation Sequencing (NGS) and data analysis (II)

IL3-independent Ba/F3 cell population establishment is described in II. Genomic DNA was extracted from the surviving Ba/F3 cell population expressing the *ERBB3* random mutation library (NucleoSpin Tissue, Macherey Nagel). A total of 24 µg of genomic DNA was used as a template for PCR amplification of ERBB3 from the samples. The NGS samples were further prepared as described in II. Sequencing libraries were produced with the Nextera DNA Flex Library Preparation Kit

(Illumina), sequenced with Illumina NovaSeq 6000, and data analyzed as described in II.

4.12 Long-read sequencing (II)

PacBio High Fidelity (HiFi) Circular Consensus Sequencing (CCS) was used to identify mutations in the same cDNA fragments, as described in II.

4.13 Protein structural analysis (I-II)

Structure preparation and molecular dynamic simulations (MDS) have been described in I and II.

4.14 Statistical analysis (I-II)

4.14.1 Cell growth assays (II)

MCF-10A growth assays and NIH-3T3 focus formation assays were statistically analyzed with an unpaired two-sample t-test to determine the differences in either absorbance values (MCF-10A) or area percent values (NIH-3T3) between the mutant and the respective wild-type ERBB control cell line. Statistical significance demonstrated as $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***). Statistics were analyzed from three to four independent experiments, as indicated in II. Wilcoxon rank sum test was used to analyze the overall Ba/F3 cell line growth differences between ERBB3 wild-type and the 14 ERBB3 variants in ligand-independent culture conditions (from eight independent experiments in total) (II).

4.14.2 Western blot signal and drug response analysis (I-II)

Western blot membranes were quantified with ImageJ (version 1.52a, I) or Image Studio Lite (version 5.2, I-II) Phosphorylation and total protein signal levels were determined as densitometric quantification. Statistics were calculated from three to five independent experiments, as indicated in I. An unpaired two-sample t-test was used for statistical analysis. Statistical significance demonstrated as $P < 0.05$.

The drug response analysis was carried out by measuring the cell viability by MTT as described in I, II, and 4.6. IC50 values for drug response were calculated to each curve as described in I and II. Data were statistically analyzed by using an unpaired two-sample t-test to determine the differences in IC50 values between the mutant and the respective wild-type ERBB control cell line. Statistical significance

demonstrated as $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***). Statistics were analyzed from three to six independent experiments, as indicated in I-II.

4.14.3 Enrichment of the mutations (II)

To identify the ERBB3 mutations that were enriched during the clonal expansion of Ba/F3 cells under IL3 depletion, fold changes were calculated. This was done as previously described in II and (Chakroborty *et al.*, 2019).

5 Results

5.1 Screening of actionable *ERBB* mutations (I-II)

5.1.1 Cancer cell line database screen identifies 62 potential predictive mutations (I)

Several publicly available databases contain sequencing and drug response data for hundreds of cancer cell lines. Taking the full potential of these data into use requires additional analyses and validation. The immense amount of data can make these processes time-consuming and laborious. Thus, a screening method for identifying potential targets from these data using simple data analysis was developed. Data for the cancer cell line database screen were obtained from Catalogue of Somatic Mutations in Cancer (COSMIC; https://cancer.sanger.ac.uk/cosmic; v71), CCLE (https://portals.broadinstitute.org/ccle/home; 24-Feb-2015 data) (Barretina *et al.*, 2012), GDSC (http://www.cancerrxgene.org; v17.3) (Yang *et al.*, 2013), and CTRP (https://ocg.cancer.gov/programs/ctd2/data-portal; v2) (Seashore-Ludlow *et al.*, 2015) as described in I.

Collectively, the three databases contained information from 1460 different human cancer cell lines (I, Fig. 1A). Sequence information (missense, nonsense, indel, truncating, or splice mutation types) was available from 1356 cell lines. Ten different ERBB TKIs were selected for drug sensitivity data collection (I, Fig. 1A).

From the 1460 different cancer cell lines, cell lines that had drug response data available for the 10 ERBB TKIs, were selected for further analysis. From this data pool, 296 unique cell lines harbored one or more *ERBB* alterations, and 997 cell lines were determined as *ERBB* wild type (I, Fig. 1A). Altogether, 412 different *ERBB* gene alterations were included in the data: 348 missense, 25 nonsense, 32 frameshift, and seven deletion, insertion or splice variant alterations.

The only measure of drug sensitivity described in all three databases was the area under the curve (AUC) (drug responses at different concentrations). Hence, it was selected for the analysis. Relative AUC (rAUC) values were calculated to normalize the drug sensitivity data for each cell line/drug/database combination, as described in I (Results). rAUC values over 1 indicated greater responses to EGFR TKIs than the responses of the cell lines harboring known EGFR driver mutations. To control for

high rAUC values associating with micromolar EC50s conferring a risk for off-target effects or not being clinically relevant, EC50 was used as a second variable for analysis of drug response (I, Fig. 1B). EC50 data was normalized by log-logistic modeling and the determined modeled EC50 (mEC50) values were used as a second variable (I).

Possible predictive mutations were identified from the population of 4685 possible cell line/drug/database combinations by determining cut-off values of > 1 for rAUC and < 1 μM for mEC50 (I, Fig. 1A and B). The cut-offs resulted in the identification of 76 mutations in 43 cell lines (I, Fig. 1C and Fig. 2; Figure 9). There were 26 mutations in the dataset that were previously identified as oncogenic mutations in the cBioPortal at the time (by OncoKB) (Cerami *et al.*, 2012; Gao *et al.*, 2013; Chakravarty *et al.*, 2017). Our analysis identified 14 (54%) of these mutations. The remaining 62 unique *ERBB* mutations (81.6%) were regarded as potentially novel (I, Fig. 2). These mutations included 18 *EGFR*, 10 *ERBB2*, 12 *ERBB3*, and 22 unique *ERBB4* genomic alterations (I, Fig. 2A; Figure 9).

To confirm the activating and predictive properties of the mutants, additional validation was required. Eleven *ERBB* mutations were selected for experimental validation *in vitro* (I, Fig. 2). The mutations included one *EGFR* mutation (Y1069C), two *ERBB2* mutations (L720P and E936K), two *ERBB3* mutations (E928fs*16 and E952Q), and four *ERBB4* mutations (A17V, L780P, G863E, and G936R). Two previously identified oncogenic mutations (EGFR G719S and ERBB2 S310F) were used as positive controls. These two mutations were known oncogenic mutations, the cell lines harboring these mutations had high rAUC values, and these mutations were not a part of the data normalization.

5.1.2 *ERBB3* iSCREAM platform identifies 18 potential activating *ERBB3* mutations (II)

To screen for activating *ERBB3* mutations in an unbiased manner from thousands of *ERBB3* mutations in parallel in a high-throughput assay (*in vitro* screen of activating mutations (iSCREAM)) (Chakroborty *et al.*, 2019), error-prone PCR was used to create a randomly mutated *ERBB3* cDNA library containing 97.3% of all theoretically possible *ERBB3* missense or nonsense mutations (as described in II). Ba/F3 cells expressing the ERBB3 mutation library together with an activator-incompetent ERBB2 V956R (Zhang *et al.*, 2006; Ward and Leahy, 2015; Pahuja *et al.*, 2018) were deprived of IL3 and from a growth-activating ligand NRG-1 to screen for activating mutations in ERBB3 (as described in II).

Next-generation sequencing from IL3-independent cell populations identified several possible activating mutations. Fold changes were calculated for each specific mutation (enrichment of a specific mutation) by comparing the read counts specific

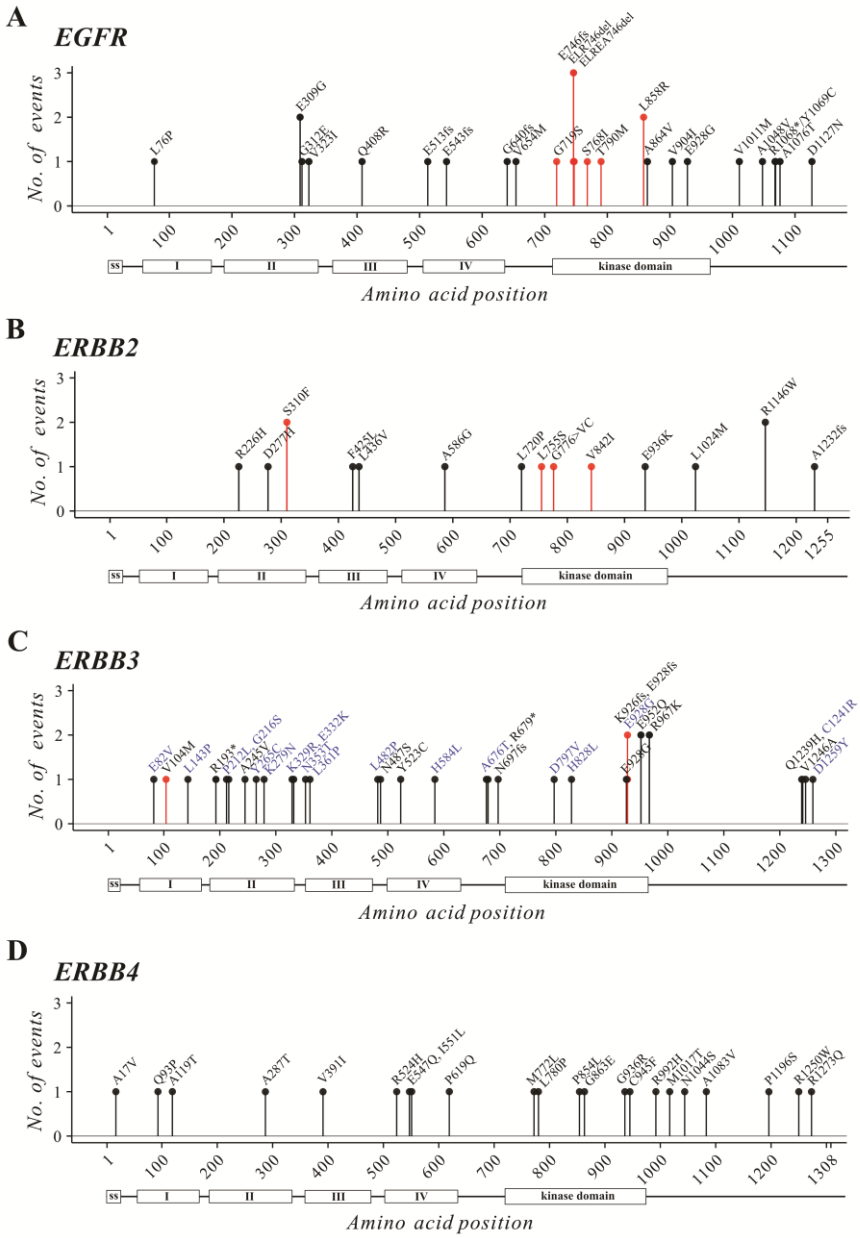

Figure 9. Putative activating *ERBB* mutations identified from the cancer cell line database screen and the *ERBB3* iSCREAM platform. The position of the lollipop on the x-axis indicates the position of the mutation in the primary sequences of the four ERBB receptors (**A**, EGFR; **B**, ERBB2; **C**, ERBB3; **D**, ERBB4). The height of the lollipop indicates the number of times the mutation was observed in the two screens. The black color of the lollipop indicates a previously unknown mutation, the red color indicates a known oncogenic mutation (OncoKB). The black color of the mutation name indicates results from the cancer cell line database screen, and the blue color results from the *ERBB3* iSCREAM platform.

for each *ERBB3* coding sequence variant in the surviving cell populations to the

original transduced IL3-dependent cell populations. Seven of the most enriched mutations were identified with a fold change of > 100 (II, Fig. 2; Figure 9). Additional 11 mutations were identified with a fold change of 25-100 (II, Fig. 2; Figure 9). These mutations were distributed relatively equally across the receptor domains (Figure 9). The majority of the most enriched mutations were located in the ECD of the receptor (ERBB3 P212L, Y265C, L361P, and L482P) (II, Fig. 2; Figure 9). ERBB3 A676T was located in the juxtamembrane region of the receptor, and two mutations were located in the kinase domain (ERBB3 D797V and E928G) (II, Fig. 2; Figure 9). Also, from the other 11 mutations, eight were located in the ECD (ERBB3 E82V, L143P, G216S, K279N, K329R, E332K, N353T, and N584L) (II, Fig. 2; Figure 9). Mutations in the kinase domain included ERBB3 H828L, and mutations in the C-terminal tail included ERBB3 C1241R and D1259Y (II, Fig. 2; Figure 9).

For the validation of activating and predictive properties of the individual mutants, 12 mutations were selected. These included all the seven most enriched mutations and five other moderately enriched mutations. The only previously characterized oncogenic mutation was E928G (Collier *et al.*, 2013; Jaiswal *et al.*, 2013), validating our approach, and it was selected to function as a positive control. Out of the other 17 potential activating mutations, the additional mutations selected for validation included mutations: P212L, Y265C, K279N, K329R, E332K, N353T, L361P, L482P, A676T, D797V, and D1259Y.

5.2 Activating EGFR and ERBB2 mutations (I)

From the cancer cell line database screen of predictive mutations, eleven ERBB mutations were selected for experimental validation *in vitro* to assess their individual activating and predictive properties (I, Fig. 2). All the mutations were transduced into Ba/F3 cells with retroviral vectors encoding the *ERBB* variants along with their corresponding wild-type receptors. Ba/F3 cells provide a cellular background devoid of significant endogenous ERBB expression (Warmuth *et al.*, 2007; Chakroborty *et al.*, 2019) and can thus be used to study activating mutations. Ba/F3 cells require IL3 for growth unless there is a RTK with an activating mutation introduced into the cell. The respective wild-type controls did not provide IL3-independent Ba/F3 cell growth in the absence of an activating ligand, while the positive controls EGFR G719S and ERBB2 S310F did (I, Fig. 3A- B). Cell lines expressing EGFR Y1069C and ERBB2 E936K were also capable of supporting IL3-independent growth in the absence of an activating ligand, thus indicating transforming potential (I, Fig. 3A-B).

5.2.1 EGFR Y1069C increases phosphorylation and demonstrates decreased association with the ubiquitin ligase c-CBL (I)

Ba/F3 cells overexpressing EGFR wild-type, Y1069C, or G719S (a positive control) were analyzed by Western blotting to study the phosphorylation status of the variants and the mechanisms by which the EGFR Y1069C variant promoted growth. As indicated, the phosphorylation of both G719S and Y1069C variants at Tyr 1086 was greater when cultured in the presence of a ligand EGF as compared to the wild-type EGFR (I, Fig. 4A). In the IL3- and EGF-independent culture condition, the wild-type cells did not survive, but both mutant variants promoted survival and demonstrated phosphorylation at Y1086 (I, Fig. 3A, Fig. 4A). Ligand stimulation enhanced downstream EGFR signaling (phospho-AKT and phospho-ERK) to a greater extent than with the wild-type receptor (I, Fig. 4A). Similar effects were observed also in NIH-3T3 cells, where the G719S and Y1069C variants demonstrated enhanced activity. However, these effects were not as prominent as compared to the Ba/F3 cell background (I, Fig. 4B).

EGFR signaling is negatively mediated by c-CBL-mediated ubiquitination and degradation of the EGFR protein (Rubin, Gur and Yarden, 2005). The binding site for c-CBL is Y1069 in EGFR (Levkowitz *et al.*, 1999). In order to test whether the Y1069C mutation disrupts the binding of c-CBL to EGFR, a co-immunoprecipitation assay was performed in NIH-3T3 cells. The analysis showed that the Y1069C mutation significantly reduced the interaction between EGFR and c-CBL (I, Fig. 4C).

5.2.2 ERBB2 E936K demonstrates enhanced transphosphorylation and increased activity of ERBB2 heterodimers (I)

Western blot analysis of the ERBB2 mutant E936K showed enhanced tyrosine phosphorylation when compared to ERBB2 wild-type (I, Fig. 5A). In the absence of both the ligand and IL3, the cells expressing the known oncogenic variant ERBB2 S310F showed enhanced phosphorylation, although the mutant E936K did not to the same extent (I, Fig. 5A). The ERBB2 S310F variant effectively promoted downstream signaling of AKT and ERK, while the E936K variant promoted only modest downstream signaling (I, Fig. 5A). When the variants were overexpressed in COS-7 cells, the E936K variant promoted ERBB2 autophosphorylation, but there was no additional activity when expressed together with ERBB3 as a heterodimer (I, Fig. 5B).

Structural analysis of E936K revealed that E936 is located at the αG helix of the kinase domain, which is conserved among ERBB kinases. In the wild-type ERBB2, the E936 (activator kinase) forms part of the dimer interface (I, Fig. 5C). Molecular dynamics simulation (MDS) of the ERBB2-ERBB2 and ERBB2-EGFR structures

revealed that the E936K mutant can form hydrogen bonds with the hydroxyl group of the S792/S784 and an additional, potentially stronger salt bridge with E717/E709 of the ERBB2/EGFR receiver kinase with the ε-amino group of K936 (I, Fig. 5D). The E936K mutant likely prolongs the duration of the activated state by strengthening the interactions between the asymmetric dimers. ERBB3 serves as an activator due to its weak kinase activity (Jura, Shan, *et al.*, 2009; Ward and Leahy, 2015), thus limiting the effects of the ERBB2 E936K mutant in the ERBB2-ERBB3 heterodimer. However, EGFR strongly prefers to function as a receiver kinase domain among the ERBB receptors (Ward and Leahy, 2015), which would suggest that the E936K mutant should have a more pronounced effect on the ERBB2-EGFR dimer. The β4-β5 loop in the E936K kinase also appeared to be more stable and less variable compared to the wild-type complex (RMSD; I, Fig. 5F-G). ERBB2 E936K mutation could lead to stronger interactions at the dimer interface based on structural analysis, but this would take place only with ERBB2-ERBB2 homodimers or with ERBB2-EGFR heterodimers.

Wild-type ERBB2 or ERBB2 E936K were transfected together with wild-type EGFR to COS-7 cells to estimate whether the ERBB2 E936K would serve as a more potent activator kinase than wild-type ERBB2. The results from four independent experiments demonstrated that ERBB2 phosphorylation was increased (two times higher phospho-ERBB2 levels; $P = 0.026$) in cells expressing both wild-type EGFR and ERBB2 E936K as compared to cells solely expressing wild-type receptors (I, Fig. 5H-I). This result suggests that ERBB2 E936K would work as a more potent activator kinase in an ERBB2-EGFR heterodimer as compared to wild-type ERBB2.

5.3 Activating ERBB3 mutations (II)

5.3.1 Enhanced growth of ERBB3 mutant cell lines (II)

ERBB3 mutations identified using the iSCREAM platform were introduced individually into Ba/F3 cell lines either alone or together with the activator-impaired ERBB2 V956R. The cells overexpressing the ERBB3 wild-type or the 12 ERBB3 mutants were cultured in the presence of IL3, in the absence of IL3, and in the presence or absence of an activating ligand NRG-1. The cells expressing ERBB3 wild-type together with ERBB2 V956R were not able to grow IL3-independently (II, Fig. 1B and Fig. 3A). Three of the ERBB3 variants (E332K, A676T, and E928G) were able to promote growth in the absence of exogenous ligands IL3 and NRG-1 (II, Fig. 3A). Three additional ERBB3 variants (K279N, N353T, and D797V) were able to promote reproducible IL3-independent growth after culturing them first with 20 ng/ml of NRG-1 for 48 hours to promote ERBB3 expression before depletion of NRG-

1. These six ERBB3 variants also demonstrated statistically significant growth ($P < 0.05$) compared to the ERBB3 wild-type (II, Fig. 4).

There were variations between the repeated experiments (II, Fig. 4), and the ERBB3 mutants P212L, Y265C, K329R, and L482P did grow IL3-independently only once out of four repetitions (after culturing the cells first with NRG-1 for 48 hours). The ERBB3 mutants L361P and D1259Y did not support IL3-independent growth at all. The delayed NRG-1-dependent growth of L361P (II, Fig. 3A) and its molecular size observed with western blotting (II, Supplementary Fig. 4B) suggested that the mutated ERBB3 receptor did not reach the cell surface. This was further confirmed with flow cytometry analysis, which demonstrated that the mutated receptor was not expressed at the cell surface (II, Supplementary Fig. 4C).

The adherent MCF-10A mammary epithelial cells overexpressing ERBB2 V956R together with wild-type ERBB3 or the 12 ERBB3 mutants were cultured in the absence of serum and in the presence or absence of 50 ng/ml of NRG-1. In the absence of serum but in the presence of NRG-1, the ERBB3 mutants K279N, E332K, N353T, L482P, A676T, and E928G demonstrated significantly more growth as compared to the cells expressing wild-type ERBB3 ($P < 0.05$) (II, Fig. 3B).

5.3.2 Enhanced focus formation of ERBB3 variants (II)

The adherent NIH-3T3 mouse fibroblast cells overexpressing ERBB2 V956R together with wild-type ERBB3 or the 12 ERBB3 variants were cultured in 3% serum for two weeks, and their ability to promote focus formation was estimated. Nine of the ERBB3 mutant cell lines demonstrated significantly more focus formation as compared to the wild-type cells ($P < 0.05$) (II, Fig. 3C-D).

5.3.3 Five of the ERBB3 mutant cell lines demonstrated enhanced growth across the three cell line models (II)

Five of the ERBB3 variants promoted significantly more growth when expressed in any of the three cell line models when compared to wild-type ERBB3 (II, Fig. 4). These were K279N, E332K, N353T, A676T, and E928G. ERBB3 variant L482P demonstrated enhanced growth compared to the wild-type cell line in MCF-10A and NIH-3T3 cells. ERBB3 variants P212L, Y265C, K329R, and D797V showed only a modest increase of growth across the three cell line models (II, Fig. 4). The mutations L361P and D1259Y did not promote enhanced growth in any of the cell line models.

5.3.4 Enhanced growth promoted by co-occurring *ERBB3* mutations (II)

Long-read sequencing was used to see whether there were composite *ERBB3* mutations that occurred *in cis* in the cell population that enriched during the clonal evolution in the screen. ERBB3 variants K279N, N353T, and E928G were the only variants to be preferably expressed alone, thus suggesting that there was no additional growth advance from other ERBB3 mutations (II, Fig. 5). The rest of the mutations (fold change ≥ 25) were found to be expressed together with two (40.0%) or three or more (32.0%) co-occurring mutations (II, Fig. 5A). The distribution of single vs. multiple mutations between those ERBB3 mutations that were enriched by 1 to 25 fold and those ERBB3 mutations that were enriched by ≥ 25 fold in the Illumina NGS analysis was significantly different ($P = 0.015$; Pearson's Chi-squared test) (II, Fig. 5A). Two co-occurring mutation pairs were further studied to see whether there would be mutation pairs that would function synergistically (II, Fig. 5B-C). Ba/F3 cells overexpressing the ERBB3 K329R+E332K double mutant were shown to enhance growth more than cells overexpressing wild-type ERBB3 or K329R or E332K mutants alone (II, Fig. 6A and C). A similar growth advantage was observed also in MCF-10A cells (II, Fig. 6B-C).

5.4 Structural analysis of the ERBB3 variants (II)

5.4.1 ERBB3 K279N regulates dimerization arm interactions (II)

Structural analysis of the ERBB3 K279N demonstrates that K279 is located within domain II, right after the dimerization arm (II, Fig. 7A-B). This location is also the beginning of the disulphide-containing module 5 of ERBB3, which contacts the dimerization arm of ERBB2. K279 contains an aliphatic part of the side chain, which interacts with the disulfide bridge of module 5. A salt bridge with D251 and a hydrogen bond with S252 are observed. A spring-like structure is observed between modules 2-7 in domain II (II, Fig. 7B). The oncogenic mutation S310F in ERBB2, located in module 6 of ERBB2, demonstrates that alterations on the "springs" can have functional effects (Diwanji *et al.*, 2021). K279N packs against P260, and there might be stabilizing interactions with T278 and V287 (II, Supplementary Fig. 9A). Strong interactions with D251 would also affect the function of the ERBB2/ERBB3 complex, and the stabilization of the complex would be more prominent.

5.4.2 ERBB3 K329R and E332K functions alone or together (II)

ERBB3 K329 is located at the beginning of extracellular globular domain III of ERBB3 (II, Fig. 7A and C). K329R has a long-length side chain and a guanidinium group and thus might increase the hydrogen bonding possibilities (II, Fig. 7C). The guanidinium group might form two hydrogen bonds to V352 (main-chain oxygen atom), and that K329R could potentially stabilize the domain in ERBB3. E332 points to the opposite direction than K329 and towards domain II (II, Fig. 7C and Supplementary Fig. 9B). A salt bridge is formed between R339 and E332. Several options are possible for the variant E332K. These include a strong hydrogen bond between K332 and the side-chain oxygen atom of S338 and the main-chain oxygen atom of G333. Another possibility includes a strong electrostatic inter-subunit link connecting K332 of ERBB3 and E280 of ERBB2 (II, Fig. 7C and Supplementary Fig. 9B). The double mutant K329R+E332K would add strength to these interactions mentioned above, which would further support the structural changes (II, Fig. 7C).

5.4.3 ERBB3 N353T may disrupt N-linked glycosylation (II)

ERBB3 N353 is one of the ten glycosylation sites in ERBB3 (Black, Longo and Carroll, 2019). A glycosylation site in ERBB3 (N418) has been shown to be important for the regulation of ERBB3 function (Yokoe *et al.*, 2007). Western blot analysis of Ba/F3 cells expressing ERBB2 V956R together with ERBB3 N353T indicated a size difference suggesting that the mutation might affect the protein's glycosylation (II, Supplementary Fig. 5B-C and Supplementary Fig. 6D). Structural analysis suggests that this mutation would eliminate the glycosylation site (II, Supplementary Fig. 9C). This loss could affect the dynamics and stability of ERBB3.

5.4.4 ERBB3 A676T may increase dimer stability (II)

Threonine phosphorylation in the JM region has effects (Red Brewer *et al.*, 2009). The A676 residue in ERBB3 is conserved among other ERBB receptors and has a threonine instead of an alanine (Guy *et al.*, 1994). Mutant versions of the T678 in EGFR have been shown to have substantial effects on the receptor function (Thiel and Carpenter, 2007). The structural analysis of the ERBB3 A676T mutation indicates that this mutant could increase the dimer stability (II, Fig. 7D-E). Possible explanations include an intrasubunit hydrogen bond between the A676T and R679 (II, Fig. 7D) or an intersubunit hydrogen bond between A676T and R669 (II, Fig. 7E). These interactions close to the plasma membrane could potentially support the kinase domain dimerization.

5.5 Drug sensitivity conferred by the ERBB mutants (I-II)

Cell lines selected for drug analysis assays included all the cell lines that promoted IL3-independent growth in the presence or absence of an activating ligand. Drug sensitivity of the *ERBB* mutant cell lines was studied by incubating the Ba/F3 cell lines expressing the wild-type *ERBB* or mutants in 96-well plates with a final concentration range of 0 to 10 μM of ERBB TKIs erlotinib (I), lapatinib (I), afatinib (I), or neratinib (I, II) or with a final concentration range of 0 to 100 μg/ml of ERBB antibodies pertuzumab (II) or trastuzumab (II) for 72 hours. The viability of cells was analyzed with the MTT assay. Ba/F3 cells infected with an empty vector, growing in the presence of IL3, served as a control for off-target toxicity.

5.5.1 EGFR Y1069C and ERBB2 E936K (I)

To study if the EGFR Y1069C and ERBB2 E936K would modify drug response, cell lines overexpressing the variants were cultured in the presence of ERBB TKIs erlotinib, lapatinib, afatinib, and neratinib for 72 hours. Internal positive controls were cell lines expressing EGFR G719S and ERBB2 S310F.

Cell lines expressing EGFR Y1069C and ERBB2 E936K both demonstrated sensitivity to the selected TKIs (I, Fig. 6). The cells expressing EGFR and cultured in the absence of the ligand were overall more sensitive to concentrations about one order of magnitude smaller as compared to cells cultured in the presence of EGF. The cell line expressing EGFR Y1069C was most sensitive to afatinib and neratinib with IC50 values at low nanomolar range (I, Fig. 6A).

With cell lines expressing ERBB2, the cells expressing ERBB2 E936K were sensitive at low or even nanomolar concentrations to afatinib and neratinib. The cell line expressing ERBB2 E936K was significantly more sensitive to afatinib in the presence of NRG-1 as compared to the cell line expressing ERBB2 wild-type (I, Fig 6B).

5.5.2 ERBB3 variants (II)

The potential to pharmacologically target the ERBB3 variants, was assessed by culturing Ba/F3 cells expressing the individual ERBB3 variants together with ERBB2 V956R in the presence of trastuzumab, pertuzumab, or neratinib. The presence of a ligand NRG-1 abrogated the responses to antibodies trastuzumab and pertuzumab, as seen with the about 1000-fold increase in IC50 values as compared to cells cultured without a ligand (II, Fig. 8A-B and Supplementary Fig. 10). The sensitivity to the TKI neratinib was similar between the different ERBB3 mutant cell lines (II, Fig. 8C; Supplementary Fig. 10). Outliers in drug responses included ERBB3 mutants P212L

and L482P, which were completely resistant to neratinib, and L361P+L482P double mutant that had similar responses to neratinib both in the presence and absence of NRG-1 (II, Fig. 8 and Supplementary Fig. 10). The L361P+L482P cells expressed low levels of ERBB3 or ERBB2 phosphorylation (II, Supplementary Fig. 7B and Supplementary Fig. 8C). The P212L and L482P mutations are located on domains II and III, on either side of the NRG-1-binding site. These mutations might result in a receptor conformation that mimics the NRG-1 bound state and leads to resistance to neratinib.

5.5.3 ERBB4 A17V and G936R (I)

Cell lines expressing ERBB4 A17V and ERBB4 G936R were cultured in the presence of the ERBB TKIs erlotinib, lapatinib, afatinib, and neratinib. The sensitivity of these cells did not significantly differ from the sensitivity of cells expressing wild-type ERBB4 (Supplementary Fig. 9C).

5.6 A summary of the ERBB mutants (I-II)

A summary of the identified novel mutant candidates describing their overall growth-promoting activities, drug sensitivities, and structural features is compiled together in Table 9.

Table 9. A summary of the ERBB mutants. Growth: activating if the activity is greater than with the cells expressing wild-type receptors in several cell line models; some activity if the activity is greater than with the cells expressing wild-type receptors in one or two cell line models. Drug sensitivity: a statistically significant difference with the indicated drug compared to the cells expressing wild-type receptors. Drug name in cursive indicates drugs that had a statistically significant difference only in the absence of a ligand. Abbreviations: NA, not available; NS, no significant difference compared to the cells expressing wild-type receptors; wt, wild-type.

MUTATION	GROWTH	DRUG SENSITIVITY	STRUCTURE
EGFR Y1069C	Activating	Afatinib, neratinib	c-CBL binding
ERBB2 E936K	Activating	Afatinib	Activator kinase
ERBB3 A676T	Activating	*Trastuzumab, pertuzumab, neratinib*	Dimer stability
ERBB3 E332K	Activating	*Trastuzumab, pertuzumab, neratinib*	Hydrogen bonding or electrostatic intersubunit link
ERBB3 E332K+K329R	Activating	*Trastuzumab, pertuzumab, neratinib*	Added strength of both mutations
ERBB3 K279N	Activating	*Trastuzumab, pertuzumab, neratinib*	Dimer formation
ERBB3 N353T	Activating	*Trastuzumab, pertuzumab, neratinib*	Glycosylation

ERBB3 D797V	Some activity	*Trastuzumab, pertuzumab, neratinib*	ATP-binding site
ERBB3 K329R	Some activity	*Trastuzumab, pertuzumab, neratinib*	Hydrogen bonding
ERBB3 L482P	Some activity	*Trastuzumab, pertuzumab*	Domain III β-sheet
ERBB3 P212L	Some activity	*Trastuzumab, pertuzumab*	Heterodimer interface
ERBB3 Y265C	Some activity	*Trastuzumab, pertuzumab, neratinib*	NA
ERBB3 D1259Y	NS	NS	NA
ERBB3 E952Q	NS	NA	NA
ERBB4 A17V	NS	NS	NA
ERBB3 E928fs*16	Less than wt	NA	NA
ERBB3 L361P	Less than wt	NS	Domain III β-helix
ERBB3 L361P+L482P	Less than wt	*Trastuzumab, pertuzumab*	NA
ERBB4 G936R	Less than wt	NS	NA
ERBB2 L720P	No growth	NA	NA
ERBB4 L780P	No growth	NA	DFG motif
ERBB4 G863E	No growth	NA	DFG motif

6 Discussion

6.1 Activating mutations can be identified from high-throughput screens

Among the thousands of cancer mutations found in patients, it can be difficult to identify the subset of predictive mutations that are found in only a small number of patients. The majority of hotspot mutations, although recurrent, are not common in any cancer type. There are only a couple of the very common hotspot mutations, such as BRAF V600E and KRAS G12 codon variations (Chang *et al.*, 2016).

The most common obstacle in identifying novel predictive mutations is the rare prevalence of the mutations in patients. This makes it harder to design clinical trials that contain enough patients. Obstacles related to *in vitro* methods include the laborious testing of hundreds or thousands of individual mutations, which takes a lot of time and resources. Thus, there is a need to develop novel methods to predict possible predictive targets among the thousands of possible mutations.

Several screening studies that provide sequencing information for either cancer cell lines or patient samples have been conducted. The majority of these results have also been focused on databases such as COSMIC and cBioPortal. This has allowed for the identification of mutation hotspots and the analysis of their predictive value. Several hotspots have been identified in the ERBB protein family, but the distribution of these mutations differs between family members. EGFR mutations are clustered in the kinase domain of the receptor and include mutations such as exon19 deletions, L858R, and T790M. ERBB2 hotspot mutations include several mutations in the kinase domain, such as L755S, V777L, and V842I, but the most common mutation observed is located in the ECD domain (ERBB2 S310F). On the other hand, ERBB3 and ERBB4 do not have any particular domains where most of the hotspot mutations are found. ERBB3 has several hotspot mutations in the ECD and kinase domain, and ERBB4 has the most evenly distributed mutations in the receptor among the ERBB receptors. Most of the identified gain-of-function mutations in ERBB receptors are missense mutations, but small deletions comprise a significant portion of the mutations. Another mutation type observed in ERBB receptors is a loss-of-function mutation, which leads to either reduced activity or complete loss of the gene product. Especially, loss-of-function mutations in the *ERBB4* gene are interesting as they have

been studied to suppress the differentiation of cancer cell lines although being able to form ligand-mediated heterodimers with ERBB2 (Tvorogov *et al.*, 2009).

This thesis studied different approaches to identify possible predictive *ERBB* mutations. The first cancer cell line database screen used three publicly available databases to identify drug response outliers in a dataset containing 4685 possible combinations of database/cell line/gene/mutation/drug (I, Fig. 1C). Normalizing the data allowed us to compare every cell line and their drug responses to the responses of clinically used predictive mutations in EGFR mutated lung cancer and to identify cell lines with similar or better responses than these positive reference controls. Our screen identified 62 potentially novel mutations dispersed between *ERBB* genes (I, Fig. 2, Figure 9), and the validation of 11 *ERBB* mutations identified two activating mutations that predicted response to ERBB TKIs.

The second approach implemented an *ERBB3* iSCREAM platform to screen for the activating potential of almost all the possible *ERBB3* mutations in parallel. Eighteen *ERBB3* mutations were identified that promoted enriched IL3-independent survival in Ba/F3 cells (with fold change ≥ 25). The activating properties of 12 *ERBB3* mutations were studied in three cell line models, and five *ERBB3* mutations activated growth across all the cell line models. The drug analysis of the ERBB3 mutant cell lines demonstrated that these ERBB3 mutant cell lines could be targeted with an ERBB TKI or an antibody (II, Fig. 8).

High-throughput screens for the identification of novel activating *ERBB* mutations have been conducted also prior to the analyses presented here, and several actionable mutations have been identified and validated (Berger *et al.*, 2016; Kohsaka *et al.*, 2017; Nagano *et al.*, 2018; Ng *et al.*, 2018; Pahuja *et al.*, 2018; Chakroborty *et al.*, 2019, 2022). These studies have identified novel cancer drivers from the vast pool of different mutant variants using various high-throughput methods, such as bar-coded library pools expressing different mutant variants. Similar cell line models have been used in these studies, and the enriched variants have been identified from the surviving cell pools by sequencing or by changes in the gene expression. Both previously known and novel growth-activating mutants have been identified from these screens. The identification of novel predictive activating mutations in this thesis indicates that it is still possible to identify rare activating *ERBB* mutations in a feasible way. For example, the oncogenic potential of ERBB3 variants has been previously overlooked, and no similar high-throughput screens trying to identify activating ERBB3 variants have been conducted. However, the use of animal models in the previous studies provides more functional information about oncogenic variants. These *in vivo* level data are missing from the experimentation described in this thesis.

6.2 Factors associating with drug sensitivity

The results from the cancer cell line database screen showed that there were also other factors in addition to sequence alterations that associated with sensitivity to ERBB TKIs. It is very well known that amplifications can predict response in certain cancer types (discussed in sections 2.5.1 and 2.5.2). Our analysis included 114 cell lines with no *ERBB* gene coding mutations but that demonstrated sensitivity to at least one ERBB TKI. Forty-five (39.5%) of these cell lines harbored copy number alterations in at least one *ERBB* gene (I, Supplementary Fig. 5). The cell lines with no *ERBB* gene coding sequence mutations, but an *ERBB* amplification, were plotted against the rAUC and mEC50 values (I, Supplementary Fig. 3 and 4). The analysis indicated that there are not many cell lines with *ERBB3* or *ERBB4* amplifications, which are sensitive to ERBB TKIs. However, there were a number of cell lines with *EGFR* or *ERBB2* amplifications that were sensitive to the pan-ERBB TKI afatinib (*EGFR* n = 14 and *ERBB2* n = 18). *ERBB2* amplification is known to associate with enhanced ERBB2-targeted sensitivity with cancer types such as breast and gastric cancer, and *EGFR* amplification is known to associate with enhanced EGFR-targeted sensitivity with colorectal cancer and HNSCC. The cell lines sensitive to afatinib identified in our analyses included breast, gastric, lung, head and neck, and esophageal cancer cells harboring amplification in *EGFR* and/or *ERBB2* (I, Supplementary Fig. 3).

The remaining 69 cell lines with sensitivity to at least one drug but no *ERBB* gene coding mutation nor an *ERBB* copy number variation indicated that there are also other factors that may explain the large number of responsive cell lines to different ERBB-targeting TKIs. It is also possible that the crude categorization of the cell lines harboring an *ERBB* gene amplification or not did prevent some additional conclusion from being made as we were not able to do quantitative analysis of the influence of the copy number variation for the observed effect. Further, the fact that there were 12 previously characterized oncogenic mutations in our screen that were not defined as activating in our analysis suggests that there might be additional concomitant oncogenic events in these cell lines that affected their drug responses (I, Supplementary Fig. 6).

6.3 Co-occurrence of mutations

Cancer samples have mutational heterogeneity, even inside the same cancer type. Differences in sequencing technology or depth, or mutation calling methods can explain some of this heterogeneity, but most of it has a biological basis coming from a different time or intensity of exposure to mutational processes (Alexandrov *et al.*, 2013, 2020; Phillips, 2018; Martínez-Jiménez *et al.*, 2020). This heterogeneity can also be seen in the cancer cell line database screen where there are differences between databases on what mutations are identified in a particular cancer cell line. Among the

thousands of cancer mutations found in cancer cell lines or patients, it is also difficult to identify those that drive cancer and those that are passenger mutations.

Several research papers from the past years clearly demonstrate that the co-occurrence of mutations or other types of alterations affects receptor activation and drug sensitivity (Skoulidis and Heymach, 2019; Saito *et al.*, 2020; Hanker *et al.*, 2021). Large-scale sequencing studies have found multiple non-random patterns of co-occurring or mutually exclusive (redundant) mutations in lung cancer (Imielinski *et al.*, 2012; Collisson *et al.*, 2014; Frampton *et al.*, 2015; Campbell *et al.*, 2016; Jordan *et al.*, 2017; Zehir *et al.*, 2017). The co-selection of these alterations suggests functional cooperation and improved signaling (Campbell, 2017; Mina *et al.*, 2017). The pre-existing MET or ERBB2 amplification in EGFR-mutant patients also associates with worse PFS with EGFR TKIs (Yu *et al.*, 2018; Lai *et al.*, 2019).

Several co-occurring mutations have also been identified in ERBB2-driven cancer, such as mutations in *PIK3CA* and *ERBB3* (Hanker *et al.*, 2013; Zabransky *et al.*, 2015; Hyman *et al.*, 2018; Smyth *et al.*, 2020). In the study of Hanker *et. al.,* the most commonly found ERBB2/ERBB3 mutation pairs were ERBB3 E928G together with either ERBB2 L755S, V777L, L869R/Q, or S310F/Y and ERBB2 S310F/Y together with ERBB3 V104L/M (Hanker *et al.*, 2021). These co-mutations were shown to increase signaling and oncogenicity. The ERBB3 E928G was also shown to promote resistance to ERBB2 antibodies, but cancer cells with co-occurring ERBB2/ERBB3 mutations were sensitive to combination treatment targeting both ERBB2 and PI3K (Hanker *et al.*, 2021).

Fifty percent of the cell lines in our cancer cell line database screen that demonstrated ERBB TKI sensitivity had one or more co-occurring mutations or amplification in other *ERBB* genes in addition to the novel *ERBB* mutation (I, Fig. 2B). These accompanying mutations might be passenger alterations or represent cooperation of oncogenic genomic alterations that could together promote cancer cell growth. Evidence supporting the cooperation hypothesis can be seen in a pan-cancer analysis of multiple mutations (Saito *et al.*, 2020). In this paper, it was shown that mutations of low functional activity might, in fact, cooperate with other mutations that are occurring *in cis* within the same oncogene. This co-occurrence was shown to result in enhanced oncogenic signaling. ERBB receptors have also been found to be among the oncogenes most frequently harboring multiple somatic mutations in clinical cancer samples (Saito *et al.*, 2020). In our cancer cell line database screen, it was shown that the activating mutations EGFR Y1069C and ERBB2 E936K had several co-occurring *ERBB* mutations in CCK81 and CTV-1 cell lines. However, our results suggested that the other *ERBB* mutations were passenger mutations. In our *ERBB3* iSCREAM platform, we were able to show a functional co-occurrence of *ERBB3* mutation K329R together with E332K that enhanced growth more than the expression of K329R or E332K alone (II, Fig. 6). The distribution of single vs. multiple mutations was also significantly different between the enriched and not enriched mutations (II, Fig. 5A).

Taken together, the data suggest that co-occurring *ERBB* variants may have functional effects.

6.4 *ERBB* mutant variants not promoting growth

Although novel activating mutations were found in the two screens, not all the validated mutations promoted growth. In the cancer cell line database screen, quite a few of the mutations seemed to have growth-activating properties. ERBB3 E952Q was found in two ERBB TKI-sensitive *ERBB2*-amplified breast cancer cell lines. We were not able to demonstrate any growth advantage for this mutation, but it has been reported to moderately enhance ERBB3 signaling in CHO cells (Pryor *et al.*, 2015) (I, Supplementary Fig. 8). There might again be differences in the cell context-dependent signaling characteristics or available dimerization partners that affect the mutated receptor's ability to activate signaling (Monsey *et al.*, 2010). The ERBB4 variants L780P and G863E were shown to be kinase-dead and failed to grow in Ba/F3 cells (I, Fig. 3D and Supplementary Fig. 9). These residues are located at the DFG motif. Based on structural analysis, the L780P mutation could drive the receptor towards an inactive state, and the G863E mutation would cause a disruption of the active state αC helix conformation as described in I. However, we did not study the ERBB4 mutations in heterodimeric complexes or in any cancer cell line models to investigate their downstream signaling and effects on oncogenicity. In previous studies, it has been demonstrated that kinase-dead ERBB4 mutations can be gain-of-function mutations when co-expressed together with ERBB2 (Tvorogov *et al.*, 2009).

In the *ERBB3* iSCREAM platform screen, only two of the validated mutations did not show a growth advantage over the ERBB3 wild-type in any of the cell models. The ERBB3 mutant L361P did not seem to be able to reach the cell surface and thus had the lowest growth-promoting activity (II, Fig. 3). The mutation was shown to co-occur with ERBB3 L482P, which had some growth-enhancing properties, but because the L361P mutation was found to be present also alone in the long-read sequencing results, we cannot completely determine it being a passenger mutation (II, Fig. 5). Moreover, the long-read sequencing results suggested that the ERBB3 D1259Y is more likely a passenger mutation, although, we cannot conclude it would not be functional together with the mutations that were not characterized (II, Fig. 5).

6.5 Drug sensitivity screens show that the identified activating mutations can predict response to ERBB inhibitor compounds

The sensitivity of the cell lines expressing the potential activating mutations was evaluated by incubating the Ba/F3 cell lines together with a selection of ERBB targeting TKIs or antibodies.

The mutations identified as activating in the cancer cell line database screen were also shown to be possible drug targets. The EGFR Y1069C was overall very sensitive to all the ERBB TKIs used in the drug screening, and the ERBB2 E936K mutation was significantly more sensitive to afatinib when compared to the wild-type expressing cell line (I, Fig. 6). The ERBB3 variants from the *ERBB3* screen were found to be the most sensitive to neratinib out of the tested compounds (II, Fig. 8).

Taken together, these drug sensitivity analyses show that the identification of previously unknown actionable mutations can reveal novel predictive mutations that can be targeted by ERBB compounds and that these mutations can be identified from different and feasible screening methods.

7 Summary/Conclusions

This thesis aimed to apply different methods to identify novel predictive *ERBB* mutations. The aims were achieved by using two different methods: a cancer cell line database screen and an *ERBB3* iSCREAM platform utilizing a cDNA library encoding randomly mutated *ERBB3* variants. These methods identified 79 novel possibly activating *ERBB* mutations, and 20 of these novel mutations were validated along with their wild-type controls and positive, known activating mutation controls. A total of six previously uncharacterized *ERBB* mutations and one double mutant were identified as actionable mutations:

1. EGFR Y1069C demonstrated enhanced phosphorylation and reduced association with the ubiquitin ligase c-CBL.
2. ERBB2 E936K promoted transphosphorylation by increasing the activity of ERBB2/ERBB2 homodimers or EGFR/ERBB2 heterodimers.
3. ERBB3 K279N demonstrated enhanced growth derived from strong interactions leading to stabilized ERBB2/ERBB3 heterodimer.
4. ERBB3 E332K demonstrated enhanced growth and effects in the hydrogen bonding possibilities. The double mutant K329R+E332K demonstrated additional enhanced growth when compared to the mutants expressed alone.
5. ERBB3 N353T demonstrated enhanced growth and a disruption of the N-linked glycosylation.
6. ERBB3 A676T demonstrated enhanced growth derived from increased dimer stability.

These results demonstrate that the two approaches can be used to identify rare activating mutations that are predictive for ERBB-targeted compounds *in vitro*.

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