HER-2/HER-3 dimerization and HER-3 point mutations in breast cancer

Master's Thesis
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Tiivistelmä

Rintasyöpä on yleinen, usein kuolemaan johtava sairaus, mistä johtuen sitä on tutkittu runsaasti viimeisten vuosikymmenten aikana. Laajamittaisen tutkimustyön ansiosta lukuisia rintasyövän syntyyn ja kehitykseen vaikuttavia solu- ja molekyylitason mekanismeja on onnistuttu selvittämään. Nykyään tunnetaan myös monia rintasyövän alatyyppejä, joista yksi tutkituimmista on HER-2 -proteiinia tavallista enemmän tuottava HER-2 -positiivinen muoto. HER-2 -positiivisen rintasyövän hoitoon on kehitetty useita lääkkeitä, joista käytetyin on monoklonaalinen vasta-aine trastutsumabi, joka tarttuu HER-2 -proteiiniin ja estää sen toimintaa. On kuitenkin havaittu, että HER-2 -reseptoriin kohdennetut, ligandista riippumatonta signalointia estävät lääkkeet, kuten edellä mainittu trastutsumabi, eivät tehoa kaikkiin HER-2 -positiivisiin kasvaimiin. Tästä johtuen uusia lääkekohteita on pyritty etsimään tehokkaampien lääkkeiden kehittämiseksi. Yksi lupaava kohde on HER-2/HER-3 -dimeeri, joka aktivoi apoptoosia estäviä sekä solujen kasvua ja jakaantumista edistäviä signalointireittejä.

Tämän opinnäytetyön tavoitteina oli määrittää HER-2/HER-3 -dimeerien määrä kahdessatoista rintasyöpä- ja yhdessä mahasyöpäsolulinjassa proximity ligation assay -tekniikkaa käyttäen, tutkia vaikuttavatko HER-3 -pistemutaatiot dimeerien muodostumiseen ja solujen lääkeherkkyyteen sekä määrittää näiden pistemutaatioiden yleisyys rintasyövässä.

HER-2/HER-3 -dimeereitä oli selvästi eniten voimakkaasti HER-2 -positiivisissa solulinjoissa, kun taas HER-2 -negatiivisissa solulinjoissa dimeereitä oli vain muutamia. Lisäksi havaittiin odotetusti, että pertutsumabi, HER-2/HER-3 -dimerisaatiota estävä lääke, vähensi dimeerien määrää useimmissa solulinjoissa. *In vitro* -lääkeherkkyyskokeet osoittivat, että dimerisaation estämisen lisäksi pertutsumabi heikensi solujen elinkelpoisuutta suurimmassa osassa HER-2 -positiivisia solulinjoja. HER-2 -positiivisten SK-BR-3 -solujen elinkelpoisuuten pertutsumabilla ei kuitenkaan ollut vaikutusta. Tämä on kiintoisa huomio, sillä *in silico* -selvityksen mukaan SK-BR-3 -solujen HER-3 -reseptori sisältää pistemutaation. Tarkempi *in silico* -tutkimus paljasti, että 1.62 prosenttia rintasyövistä sisältää HER-3:n aminohappokoostumusta muuttavia pistemutaatioita. Jos käsitellään eri alatyyppejä erikseen, 3.36 prosenttia HER-2 -positiivisista tapauksista sisältää pistemutaatioita HER-3 -proteiinissa, kun taas vain 1.42 prosenttia HER-2 -negatiivisista tapauksista sisältää HER-3 -pistemutaatioita. Tämä havainto on mielenkiintoinen, ja HER-3 -pistemutaatioiden merkitystä HER-2 -positiivisen rintasyövän synnylle ja kehitykselle onkin syytä tutkia yksityiskohtaisesti tulevaisuudessa. Lisäksi on tärkeää selvittää, johtuuko SK-BR-3 -solujen pertutsumabiresistenssiys pistemutaatioista HER-3 -reseptorissa. Jos näin on, mutaatioiden olemassaolo on otettava huomioon kehitettäessä uusia, HER-3 -reseptoriin kohdennettavia lääkkeitä.

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Abstract

Breast cancer is a common disease and an important cause of mortality in women. Breast cancer has been extensively studied during the last few decades, which has led to the development of potent drugs and to the identification of several breast cancer subtypes. HER-2 positive breast cancer is one of the most well-known subtypes, and drugs which directly target this receptor and inhibit the ligand independent signaling pathways have been developed. Some HER-2 positive breast cancers, however, are insensitive to these drugs, and this has led to the need to develop alternative treatment methods and to find new drug targets. HER-2/HER-3 heterodimers are among the most promising new targets. These dimers activate downstream signaling pathways such as PI3K/Akt, which inhibit apoptosis and promote cell proliferation.

The aims of this thesis were to quantitate the amount of HER-2/HER-3 dimers in twelve breast cancer cell lines and in one gastric cancer cell line using proximity ligation assay technique, to examine do point mutations in the HER-3 receptor affect HER-2/HER-3 dimerization and drug response *in vitro*, and to determine the prevalence of these mutations in breast cancer using *in silico* methods.

As expected, HER-2/HER-3 dimers were abundant in most HER-2 positive cell lines, while HER-2 negative cell lines had only a few dimers. Additionally, the addition of pertuzumab, a HER-2/HER-3 dimerization inhibitor, decreased the amount of dimers in most tested cell lines. *In vitro* drug sensitivity assay confirmed that pertuzumab alone is capable of preventing the growth of most HER-2 positive cell lines. However, despite the abundance of HER-2/HER-3 dimers, SK-BR-3, BT-474, and ZR-75-30 cells were insensitive to pertuzumab. Curiously, *in silico* evaluation revealed that one of these three cell lines, SK-BR-3, contains a point mutation in the HER-3 receptor, which might explain the observed resistance to pertuzumab. Further *in silico* study revealed that 1.62% of all breast cancers contain nonsynonymous point mutations in the *ERBB3* gene which encodes the HER-3 protein. When considering different cancer subtypes, 3.36% of the 119 HER-2 positive samples studied contained point mutations in *ERBB3*, while only 1.42% of the 843 HER-2 negative samples contained point mutation in the gene. The fact that HER-2 positive breast cancers contain twice as many point mutations as other breast cancer subtypes is interesting, and therefore the significance of these mutations to the onset and development of HER-2 positive brest cancer should be carefully evaluated in the future. Additionally, it is important to investigate if the pertuzumab resistance of SK-BR-3 cells is caused by the HER-3 point mutation. If this is the case, the presence of HER-3 point mutations should be taken into account when new HER-3 targeted drugs are developed.

Acknowledgements

This thesis was carried out in Jorma Isola's Cancer Biology Group at BioMediTech, University of Tampere. Jorma was also my supervisor, and I would like to thank him for this opportunity to be a part of an intersting and meaningful research project and his guidance throughout this endeavor.

Like any scientific work nowadays, this thesis cannot be considered as a sole creation of the author. I have been, as Isaac Newton once said, standing on the shoulders of giants: it has been a priviledge to be able to quote the pioneering works of nearly legendary cancer researchers such as Dennis Slamon. Yet, even more important have been the support and assistance which I have received from all the people working with me during this project. I would like to especially thank M.Sc Satu Luhtala for her patience and great advice, and Mrs. Sari Toivola, probably the best laboratory techinician in the world, for helping me whenever I needed anything.

I thank my family for their support, and give my parents a special mention for passing me down genes that made this work possible. I would also like to thank my friends for the great university years and memories that I will never forget.

Tampere, October 2014

Jani Sarin

Contents

Abbreviations

Ab	antibody
ADCC	antibody-dependent cell-mediated cytotoxicity
Akt	protein kinase B
AP	activator protein
BAD	bcl-2-associated death promoter
BRCA	breast cancer 1, early onset
Calcein AM	acetomethoxy derivate of calcein
CCLE	cancer cell line encyclopedia
COSMIC	catalogue of somatic mutations in cancer
DAPI	4',6-diamidino-2-phenylindole, a fluorescent dye binding strongly to A-T rich regions of DNA
DNA	deoxyribonucleic acid
ECD	extracellular domain
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor, another name for HER-1
ERBB	another name for human epidermal growth factor receptor
FoxO	forkhead box class O
HER	human epidermal growth factor receptor
Ig	immunoglobulin
IKK	IκB kinase complex
JAK	janus kinase
MAPK	mitogen activated protein kinase
MEK	mitogen activated protein kinase kinase
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
MUC-1	mucin 1, cell surface associated
NRG	neuregulin
p85-sHER-3	a truncated, soluble form of HER-3
PBS	phosphate buffered saline
PDB	protein data bank
PI(3,4)P2	phosphatidylinositol-3,4-bisphosphate
PI(3,4,5)P3	phosphatidylinositol-3,4,5-triphosphate
PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
PLA	proximity ligation assay
PLCγ	phospholipase C-gamma
RNA	ribonucleic acid
SNP	single nucleotide polymorphism
Sp1	specificity protein 1
STAT	signal transducer and activator of transcription
TCGA	the cancer genome atlas

1. Introduction

For a long time, cancer has been a major health problem worldwide, and yet the incidence of this group of related diseases is increasing: in 2012 there were approximately 14 million new cancer cases, and this number is estimated to grow to 24 million in the following 20 years (Ferlay et al. 2013). Even though there are over 200 different types of cancer, only six cancer types (lung, breast, colorectum, prostate, stomach, and liver; non-melanoma skin cancer excluded) constitute 55% of all new cancer cases (Ferlay et al. 2013).

1.68 million new breast cancers in the world were diagnosed in 2012, making it the second most common cancer in both sexes with 11.9% of all new cases (Ferlay et al. 2013). In women, breast cancer is clearly the most common cancer with 25.2% of all new cases (Ferlay et al. 2013). Since breast cancer is such an important disease, it has been extensively researched for decades: a simple PubMed search with terms "breast cancer" yields over 282 000 results. First of these publications have been published in the 19th century.

Due to the extensive research, new treatment methods and drugs have been developed, and diverse molecular mechanisms affecting the onset and development of breast cancer have been discovered in the past decades. Several prognostic and predictive markers have been established, the most important and widely researched molecular factors being HER-2, estrogen receptor, and progesterone receptor (Cianfrocca and Gradishar 2009, Köninki 2010, Bertos and Park 2011, Gannon et al. 2013, Rivenbark et al. 2013). Breast cancer is categorized to four different main subtypes depending on which of these receptors, if any, are overexpressed (Norum et al. 2004, Köninki 2010, Bertos and Park 2011). This categorization is important as HER-2 positive tumors, for example, require different treatment methods when compared to tumors of other categories, such as the triple negative subtype (Norum et al. 2004). It is therefore vital to identify the molecular variety in all new breast cancer cases in order to choose the right therapy with high therapeutic efficacy and minimal side effects (Norum et al. 2004, Köninki 2010, Rivenbark et al. 2013).

It is noteworthy that even though HER-2, estrogen receptor, and progesterone recetor are the most well-known molecular factors associated with breast cancer, they are not the only ones. One of the less researched molecular factors is HER-3, a close relative to HER-2 (Hamburger 2008). HER-3 forms dimers especially with HER-2 and activates PI3K/Akt pathway, which inhibits apoptosis and promotes cell proliferation (Chang et al. 2003, Hsieh and Moasser 2007).

The aim of this thesis is to determine the abundance of HER-2/HER-3 dimers in HER-2 positive breast cancer cell lines and to determine how the amount of these dimers correlates with the drug response to pertuzumab, a HER-2/HER-3 dimerization inhibitor. Additionally, it has been suggested that point mutations in the HER-3 receptor could be rather common, and the abundance of these mutations in breast cancer is evaluated *in silico* (Jaiswal et al. 2013). The possible effect of these mutations to HER-2/HER-3 dimerization is also studied.

2. Review of the literature

2.1. Origin of breast cancer

As mentioned before, breast cancer is the most common cancer in women. There are several different subtypes of human breast cancer, all of which have distinct clinical course, therapeutic response, and morphology (Norum et al. 2004, Köninki 2010, Bertos and Park 2011). Due to this heterogeneity, a single mechanism of breast cancer development has been hard to decipher. Previously it was thought that breast cancer development is a multi-step process following a single pathway: the disease develops over an extended period of time, beginning with dysplasia, progressing to invasive carcinoma and finally to metastatic disease (Falkenberry and Legare 2002, Ye et al. 2004). New evidence, however, suggest that this kind of systematic multi-step model is at least partially too simplistic, and that the development of breast cancer involves a complex series of random mutations which, sooner or later, lead to an invasive disease and possible cancer stem cell formation (Köninki 2010). According to recent studies, these random mutations do not necessarily lead to a progress from well differentiated through moderately differentiated to undifferentiated tumors (Köninki 2010).

Mutations in tumor suppressor genes such as *BRCA1* are thought to be important in the development of breast cancer (Lee and Muller 2010). Tumor suppressor genes encode proteins that are involved in cell cycle regulation, apoptosis, angiogenesis, DNA repair, and generally in many important cellular processes inhibiting tumor formation (Köninki 2010, Lee and Muller 2010). Point mutations, chromosomal deletions or epigenetic changes can inactivate tumor suppressor genes and lead to tumor formation (Köninki 2010). Tumor suppressor gene mutations are often accompanied with mutations in proto-oncogenes (Osborne et al. 2004). These genes and their protein products are usually involved in cellular proliferation and apoptosis, enhancing the former and inhibiting the latter (Osborne et al. 2004). Protein products of proto-oncogenes are needed in normal cells, but if these genes are mutated to oncognes, normal cellular processes and downstream signaling pathways may be disturbed and a cell may start to grow and proliferate uncontrollably (Todd and Wong 1999).

Genes coding the members of the human epidermal growth factor receptor (HER) family are among the best known and most important oncogenes associated with breast cancer (Osborne et al. 2004).

2.2. Human epidermal growth factor receptors

The human epidermal growth factor receptor family consists of four membrane-bound receptor proteins: HER-1 (EGFR), HER-2, HER-3, and HER-4 (Olayioye et al. 2000, Hsieh and Moasser 2007). These receptors are expressed in various tissues of mesenchymal, epithelial and neuronal origin (Köninki 2010). Each receptor of the HER family has an extracellular, transmembrane, and intracellular domain (Burges et al. 2003). The extracellular domains of HER-1, HER-3, and HER-4 bind ligands such as epidermal growth factor (EGF) molecules (Burges et al. 2003). The extracellular domain also contains a dimerization subdomain (more detailed information in section 2.6.). The transmembrane domain attaches a receptor to cell membrane (Burges et al. 2003). The intracellular protein tyrosine kinase domain activates downstream signaling pathways (Olayioye et al. 2000). These pathways affect growth, survival, and proliferation of a cell.

In normal cells the activation of HER family receptors and consequential cellular signaling are strictly regulated. If this regulation does not work as it should, signaling pathways may become overactive and a cell may become malignant (Olayioye et al. 2000). This leads to various alterations of transport pathways and overall changes in biological behaviour of the cell. It has, for example, been reported that even though the receptor tyrosine kinases are primarily membrane proteins, the members of HER family can be abundant in the nucleus of cancer cells (Wang et al. 2010).

2.3. HER-2 in breast cancer

Human epidermal growth factor receptor 2 (HER-2) is a widely researched receptor of the HER family as well as an important target of therapy in breast cancer. HER-2 is encoded by the *ERBB2* gene which is located in chromosome 17q (Schecter et al. 1984, King et al. 1985). It has been estimated that the *ERBB2* gene is amplified in 10–25% of breast cancers (Seshadri et al. 1993, Andrulis et al. 1998, Owens et al. 2004, Köninki 2010, Harbeck et al. 2013). The estimated proportion of breast cancer with *ERBB2* amplification has steadily declined during the last few decades, and the percentage is probably closer to the lower end of the scale (Köninki 2010). A normal cell has only two copies of the *ERBB2* gene, whereas a HER-2 positive cell may contain up to 100 copies of the gene (Perez and Baweja 2008). Amplification of the *ERBB2* gene leads to HER-2 protein overexpression and consequently to excessive activation of the downstream signaling pathways (Slamon et al. 1987). HER-2 overexpression is associated with a more aggressive clinical course and poorer prognosis when compared to breast cancer subtypes not overexpressing HER-2 (Perez and Baweja 2008).

2.4. The *ERBB3* gene and the HER-3 receptor

2.4.1. The *ERBB3* gene

Human epidermal growth factor receptor 3 (HER-3) is coded by the *ERBB3* gene, which is located on chromosome 12q13.2 (Sithanandam and Anderson 2008, Koutras et al. 2010). The gene is 23.2 kb in size and it consists of 28 exons (Sithanandam and Anderson 2008). The extracellular domain coding sequence has 43–45% homology and the intracellular domain coding sequence has 60–63% homology with the *ERBB2* gene (Sithanandam and Anderson 2008). A full-length *ERBB3* gene transcript consists of 4080 nucleotides and 1342 codons (Sithanandam and Anderson 2008). Additionally, there are five truncated *ERBB3* transcripts which range from 1.4 kb to 2.3 kb in size (Sithanandam and Anderson 2008). The truncated transcripts code for both secreted proteins and membrane-bound proteins (Sithanandam and Anderson 2008). It has been shown that secreted transcripts of the *ERBB3* gene, especially a protein called p85-sHER-3, bind neuregulins (NRGs, HER-3 ligands) and reduce the ligand activity of NRGs in breast cancer cells (Sithanandam and Anderson 2008).

The promoter region of the *ERBB3* gene is GC rich (65%) and it does not contain a TATA box (Sithanandam and Anderson 2008). Instead, five potential nuclear factor binding sites have been identified in the promoter region. It is not precisely known which transcription factors are involved in the activation of the *ERBB3* gene, but it is believed that the Sp1 transcription factor is involved in the process as it binds to upstream of intron 1 enhancers (Sithanandam and Anderson 2008). Additionally, overexpression of AP transcription factors as well as the presence of α6β4 integrin have been shown to increase the *ERBB3* mRNA levels in breast cancer cells (Sithanandam and Anderson 2008). Estrogen, on the other hand, decreases *ERBB3* mRNA levels in estrogen receptor-positive breast cancer cell lines (Bates and Hurst 1997, Revillion et al. 2003, Sithanandam and Anderson 2008).

2.4.2. Overview of the HER-3 receptor

Human epidermal growth factor receptor 3 (HER-3) is a widely expressed protein in human tissues. It is detected in brain, liver, kidney, spinal cord, prostate, lung, and mammary gland (Sithanandam and Anderson 2008). HER-3 could be considered the little brother of HER-2: even though HER-3 is not as widely researched as HER-2, it is increasingly investigated for its role in the onset and development of cancer. HER-3 receptor is activated by ligand (neuregulin-1 (NRG-1) and neuregulin-2 (NRG-2)) binding (Stern 2008). The intracellular kinase domain of HER-3 is inactive, and it is therefore unable to initiate downstream signaling pathways on its own (Hsieh and Moasser 2007, Koutras et al. 2010). However,

HER-3 can dimerize with other receptors, most importantly with HER-2, to increase downstream signaling (Koutras et al. 2010). HER-3 receptors have been estimated to be overexpressed in 20–30% of invasive breast carcinomas (Koutras et al. 2010).

Such as all members of the HER family, a single HER-3 receptor has an extracellular domain, a transmembrane domain, and an intracellular domain. HER-3 receptor consists of 1342 amino acid residues. The atomic mass of HER-3 is 180 kDa, of which up to 30 % consists of glycosyl groups (Sithanandam and Anderson 2008).

2.4.3. Extracellular domain of HER-3

The extracellular domain of HER-3 consists of 624 amino acid residues which form four subdomains: I, II, III, and IV (Cho and Leahy 2002, Sithanandam and Anderson 2008). Domain I consists of 190 residues and it has a β-helical, small disulfide-containing modules containing structure. Domain II also consists of ~190 amino acid residues but it exists in a more extended conformation when compared to domain I. Domain III contains 120 residues and its structure is similar to domain I. Domain IV consists of 120 residues and it is an extended repeat of seven small disulfide-containing modules, similar to those in domain II (Cho and Leahy 2002). The PDB structure of the extracellular domain is shown in figure 1.

When a ligand is not bound to HER-3, domains II and IV interact with each other and the residues 242–259 of domain II form a β-hairpin loop which keeps the receptor in closed conformation preventing the dimerization arm of domain II from interacting with other receptors (Sithanandam and Anderson 2008). Domains I and III are responsible for ligand bindind and, unlike in HER-1, domain III ligand binding is dominant in HER-3 (Sithanandam and Anderson 2008). When the receptor binds a ligand, domains I and III form a rigid structure and the domain II expands and interacts with the dimerization domain of another ligand-bound member of the HER family to form dimers (Sithanandam and Anderson 2008). Domain IV interacts with domain II, enhancing the ligand binding affinity of the receptor 20- to 40-fold (Cho and Leahy 2002). Domain IV, however, is not directly involved in the dimerization process (Cho and Leahy 2002).

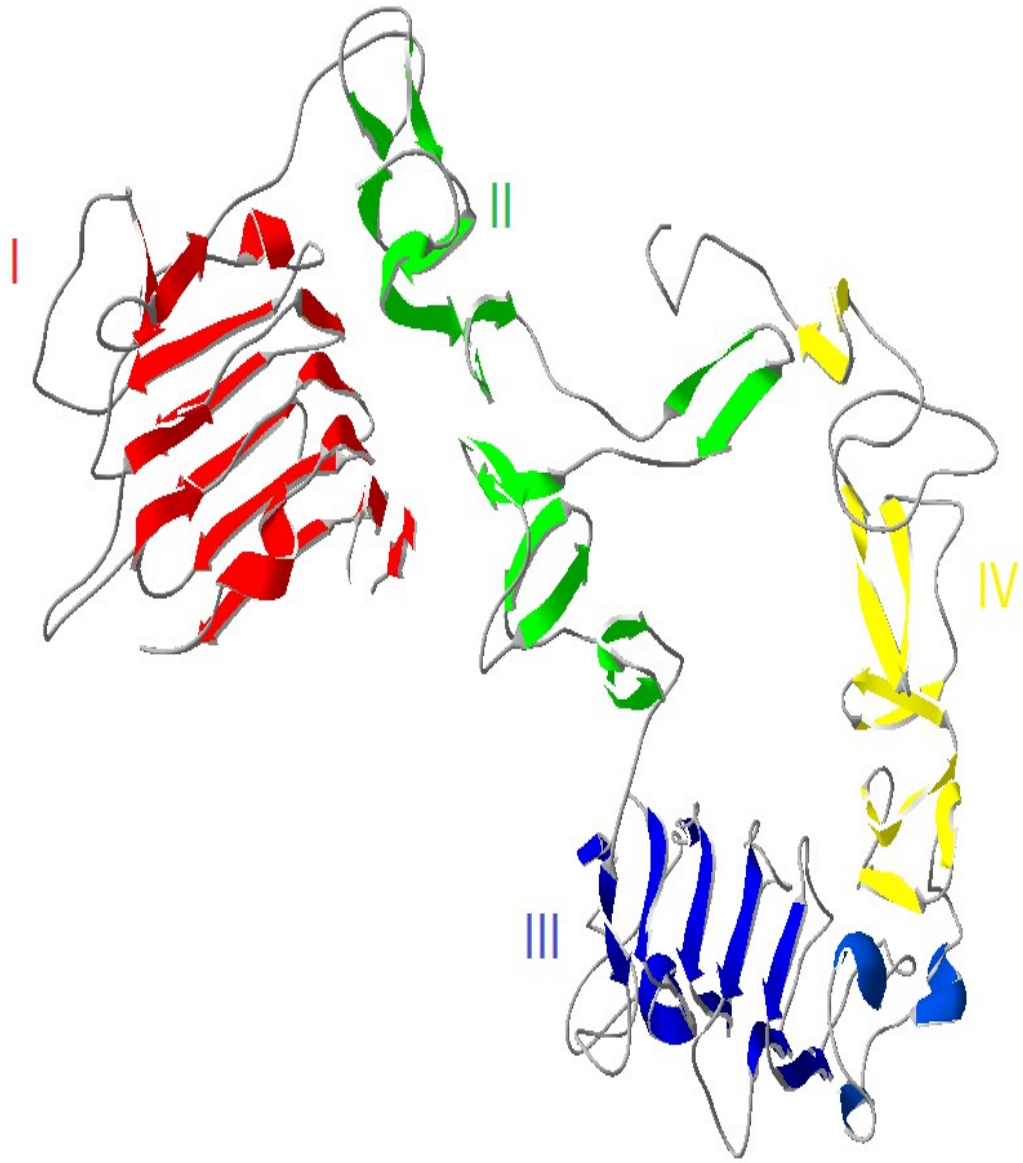

Figure 1. The PDB structure (1M6B) of the extracellular domain of HER-3. Domains I (red) and III (blue) have similar structure, and so do domains II (green) and IV (yellow).

2.4.4. Transmembrane region of HER-3

The transmembrane domain of HER-3 is a single helical structure that contains 21 amino acid residues. It has one GXXXG consensus sequence that enhances the efficiency of receptor homodimerization (Sithanandam and Anderson 2008). Other members of the HER family contain two GXXXG concensus sequences, and due to this it is believed that HER-3 is less able to form homodimers when compared to for example HER-2 (Sithanandam and Anderson 2008). The relative inability of HER-3 to form homodimers is considered to promote heterodimerization especially with HER-2 (Sithanandam and Anderson 2008). The PDB structure of the transmembrane region of a homodimer formed by two HER-3 receptors is shown in figure 2.

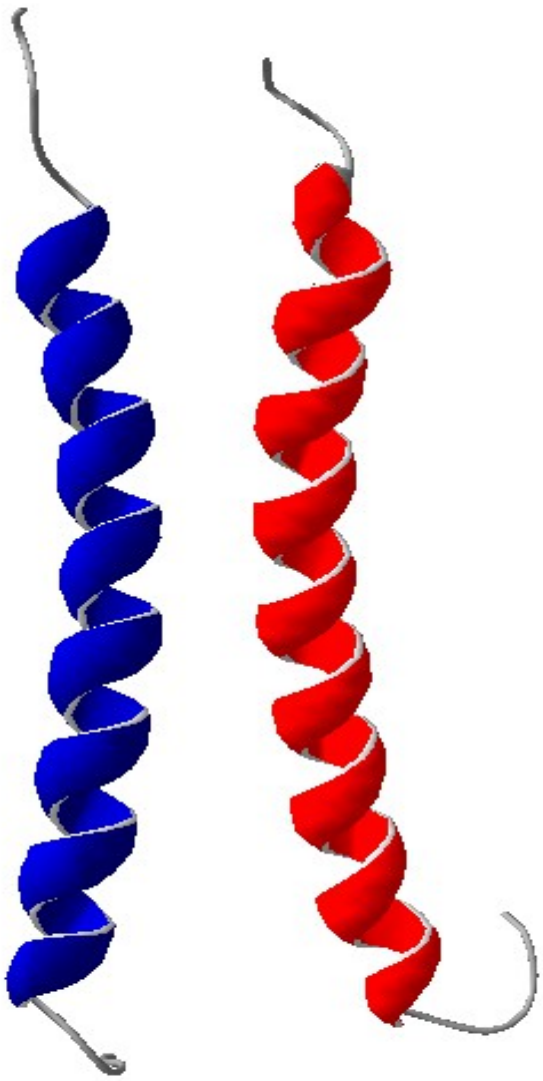

Figure 2. The PDB structure (2L9U) of the transmembrane region of a homodimer formed by two HER-3 receptors.

2.4.5. Intracellular region of HER-3

The intracellular domain of HER-3 consists of 697 amino acid residues, of which 268 form the cytoplasmic kinase domain. The amount of amino acids in different HER receptors vary from 1210 (HER-1) to 1342 (HER-3), but the amount of amino acids (268) in the kinase domain is conserved in all the members of HER family. 192 of the total 268 amino acid residues are identical in HER-1, HER-2 and HER-4. Of these 192 identical residues, 144 are conserved in HER-3 (figure 3).

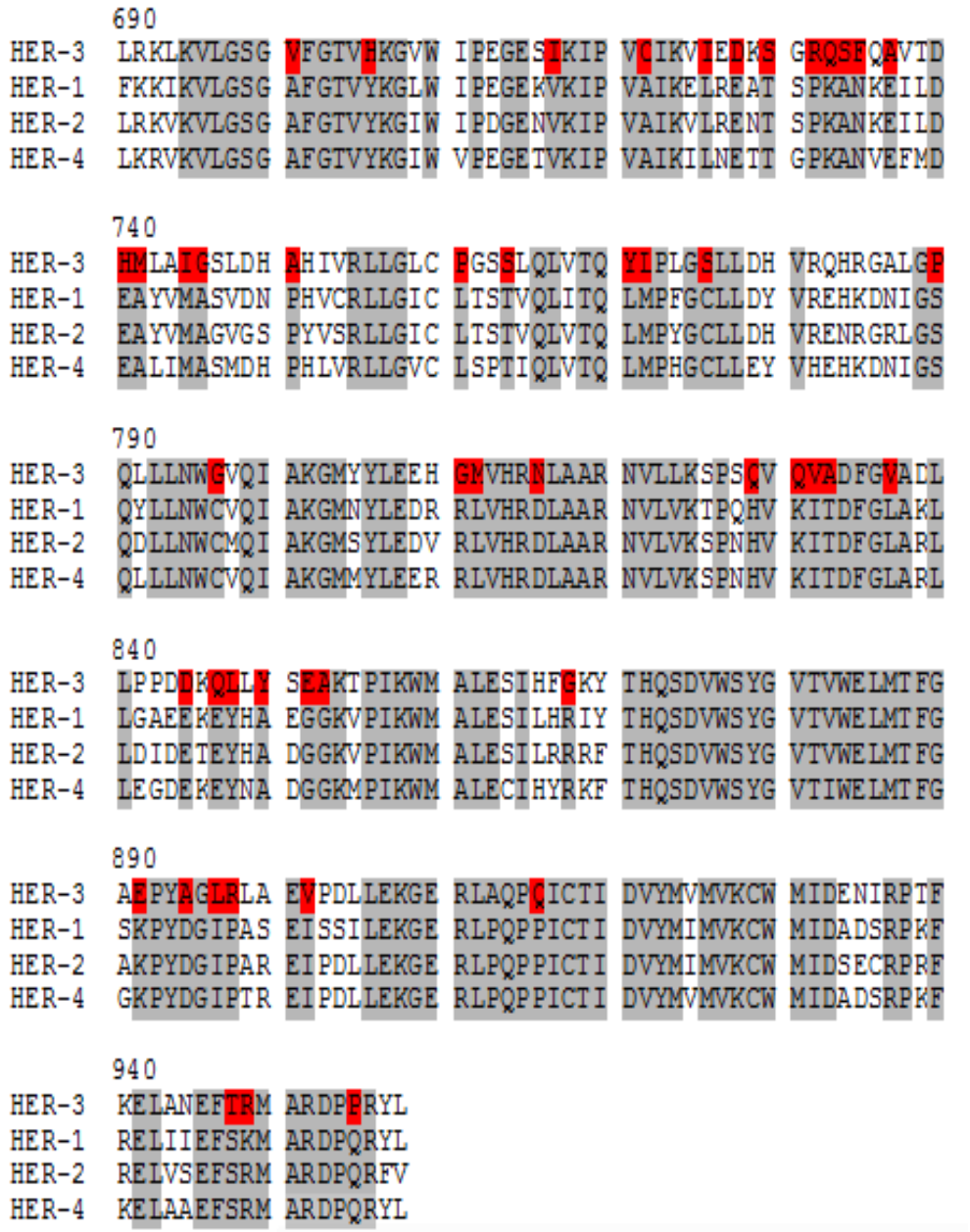

Figure 3. The sequences of kinase domains of HER-1, HER-2, HER-3, and HER-4 aligned. Amino acids that are identical in all receptors (except in HER-3 in some cases) are highlighted in gray. Red highlightion shows the HER-3 residues which are different from the conserved residues of other receptors. Sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank/).

Because the amino acid composition of the kinase domain of HER-3 differs significantly from other members of the HER family, and because the intracellular domain of HER-3 does not become autophosphorylated, the receptor is generally considered to have a very low kinase activity (100-fold reduced when compared to other members of the HER family) (Sithanandam and Anderson 2008, Shi et al. 2010). Some of the amino acid changes are more significant than others, and especially the change of glutamate 740 of other kinases to histidine in HER-3, the change of aspartate 815 of other kinases to asparagine in HER-3, and the change of asparagine of other kinases to phenylalanine in HER-3 are thought to be important inactivating factors. E740 is indirectly involved in ATP binding, D815 functions as

a catalytic base in protein kinases deprotonating substrate hydroxyl group, and mutated F734 in HER-3 provides hydrophopic interactions that stabilize the inactive conformation of kinase domain (Jura et al. 2009, Shi et al. 2010).

Even though the dysfunctional kinase activity of HER-3 has been established, it is not fully understood how HER-3, then, is able to enhance the phosphorylation of HER-2 in a HER-2/HER-3 dimer (Sithanandam and Anderson 2008). One explanation to this could be that HER-3 recruits another cytoplasmic kinase that is responsible for the phosphorylation of HER-2 (Sithanandam and Anderson 2008). This scenario, though, is unlikely, and it is more probable that allosteric interactions between kinase regions of HER-2 and HER-3 are responsible for the phosporylation and subsequent activation of HER-2 (Sithanandam and Anderson 2008). It has been suggested that even though HER-3 is catalytically inactive, it can serve as an activator of other members of the HER family (Slamon et al. 1987, Jura et al. 2009). This involves the formation of asymmetric dimers between the kinase domains of HER-3 and the kinase domain of another member of the HER family (Zhang et al. 2006, Jura et al. 2009). One of the kinase domain functions as an activator and the other functions as a receiver. The activator kinase plays a role similar to that of cyclin in cyclin-dependent kinases, stimulating autophosphorylation and activation of the receptor kinase (Jura et al. 2009). Thus, even though HER-3 cannot function as the receiver or become autophoshorylated, it can function as the activator kinase and activate other members of the HER family (Jura et al. 2009).

A schematic diagram of the intracellular kinase domain of a HER family receptor is shown in figure 4. Figure 5 shows the PDB-structure of intracellular domain of HER-3.

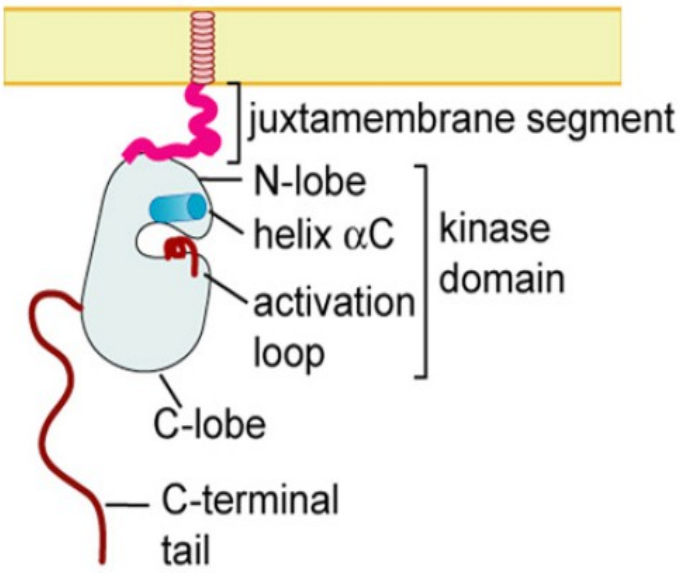

Figure 4. A schematic diagram of the intracellular kinase domain of a HER family receptor. The juxtamembrane segment attaches the kinase domain to the transmembrane domain of the receptor. Juxtamembrane domain also interacts with the C-lobe and participates in dimer formation when a ligand is bound to the extracellular domain of the receptor. C-lobe, N-lobe, helix αC, and activation loop form the actual kinase domain, which phosphorylates the C-terminal tail. Helix αC is an important element in kinase regulation. When the receptor is in inactive conformation, activation loop is tightly packed inside the active site of helix αC. When the receptor is activated, conformations of both helix αC and activation loop change and the kinase domain becomes able to phosphorylate its target. C-terminal tail contains three phosporylation sites which mediate the activation of downstream signaling pathways. Adapted from Jura et al., (2009).

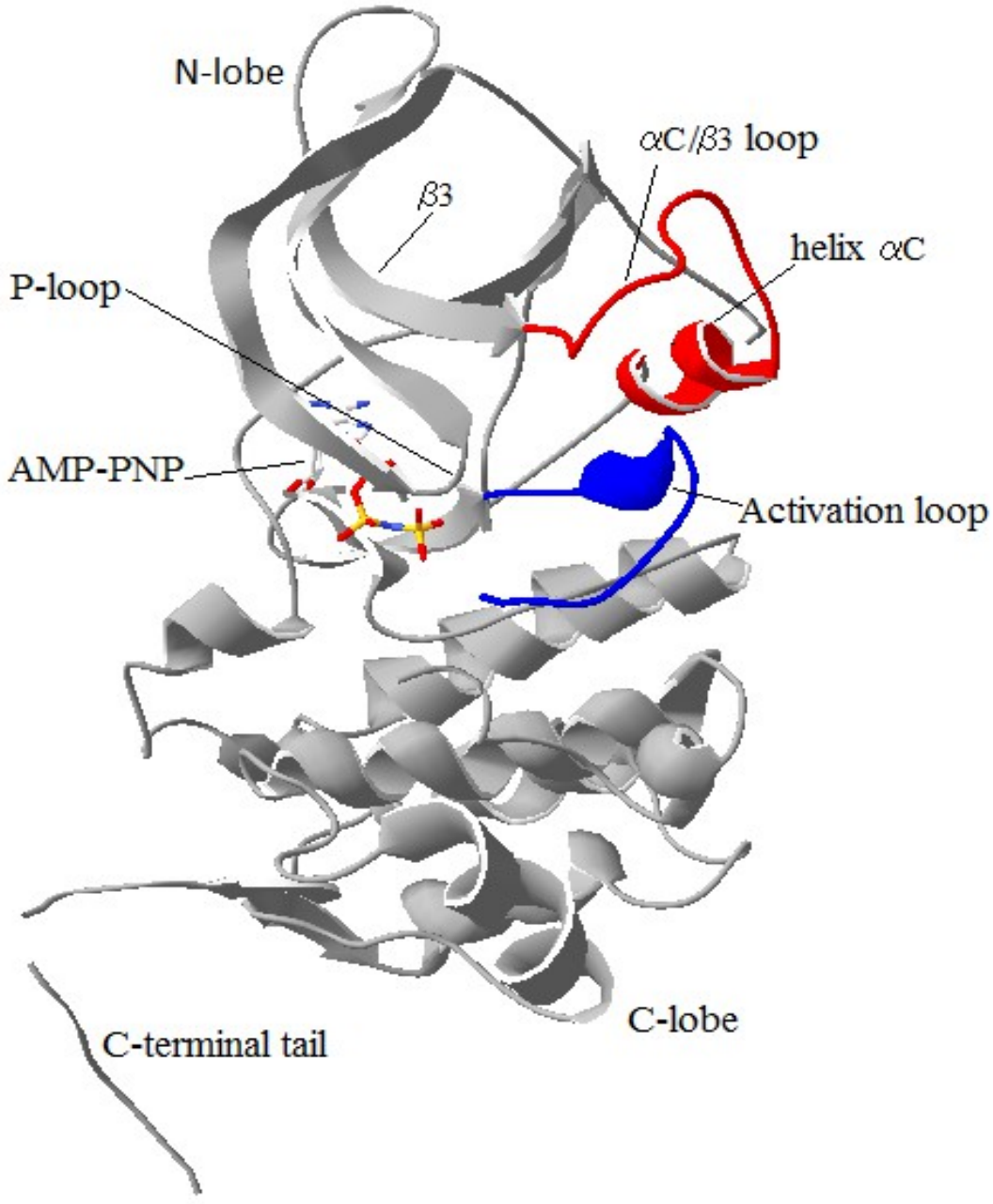

Figure 5. PDB-structure (3KEX) of the intracellular domain of HER-3. Helix αC, activation loop, N-lobe, and C-lobe form the actual kinase domain. In HER-3, αC/β3 loop contains mutated F734, which stabilize the inactive conformation of activation loop and helix αC. AMP-PNP is a nonhydrolyzable analogue of ATP which, in active kinases, is used to phosphorylate the C-terminal tail. P-loop is important to the correct localization of ATP. C-lobe of the activator kinase (e.g. HER-3) can interact with the N-lobe of the receiver kinase (e.g. HER-2) and help it to become autophosphorylated.

2.4.6. HER-3 receptor in breast cancer

Even though HER-3 alone or as a homodimer is inactive, it can active several downstream signaling pathways (PI3K/Akt, PLCγ/PKC, Jak/Stat, and MEK/MAPK) through heterodimerization with other members of the HER family, especially with HER-2 (Metzger-Filho et al. 2013, Ma et al. 2014). This heterodimerization, discussed in more detail in section 2.6.2., is important to breast cancer development: it has for example been observed that the existence of HER-3 receptors is required to maintain the tyrosine kinase activity of HER-2 (Ma et al. 2014).

It is common that the expression level of the *ERBB3* gene is increased in breast cancer (Ma et al. 2014). It has been evaluated by real-time PCR that *ERBB3* mRNA levels in different breast cancers show 100-fold variation, and that the expression level is on average 46% higher in cancer cells than in normal cells (Sithanandam and Anderson 2008). HER-3 protein, on the other hand, is detectable in 50–70% of human breast cancers, and the levels of the protein are higher in cancer cells than in normal cells in 18–29 % of the cases (Sithanandam and Anderson 2008). Overexpression of HER-3 has been associated with larger tumor size and with increased metastasis and recurrence (Ma et al. 2014). However, reports have been controversial: while most of the studies support the above mentioned obsevations which lead to poorer prognosis, some have reported that HER-3 overexpression actually improves prognosis (Ma et al. 2014). A few theories have been proposed to explain these contrary observations. One of these theories tries to explain why, unlike expected, HER-3 overexpression can improve the prognosis of breast cancer: the *ERBB3* gene can be expressed in an alternatively spliced forms generating a truncated, soluble isoform of the HER-3 protein (p85-sHER-3) (Ma et al. 2014). This isoform can bind to NRG-1 and NRG-2 with high affinity, thereby blocking the binding of these ligands to the HER-3 receptors located on the cell surface. Increased expression of the *ERBB3* gene leads to elevated amounts of p85-sHER-3 and consequently to increased blockage of ligand binding. When ligand binding is blocked, downstream signaling cannot occur (Ma et al. 2014). According to second theory, the localization (on the cell membrane, in the cytoplasm, or in the nucleus) of HER-3 receptors can affect their activity (Ma et al. 2014). Thus, higher concentration of the molecules does not necessarily correlate with the activity of downstream signaling pathways. If most of the receptors are located in the cytoplasm or in the nucleus, they can't bind their ligands or form dimers with other receptor tyrosine kinases, and consequently downstream signalling pathways cannot be activated. Several factors, such as the concentration of NRGs, can affect the cellular location of HER-3 receptors and therefore have an impact on the HER-2/HER-3 heterodimer mediated signaling (Ma et al. 2014).

2.5. Point mutations in the *ERBB3* gene and in the HER-3 protein

2.5.1. Point mutations in the *ERBB3* gene

528 single nucleotide polymorphisms (SNP) in the *ERBB3* gene have been found (Raghav and Sharma 2013). 352 of these mutations are located in introns, while the rest 176 are located in exons. Of the exon mutations 79 are nonsynonymous missense SNPs, while 37 are synonymous mutations (Raghav and Sharma 2013). Rest of the exon SNPs consist of frameshift mutations, mutations in the untranslated regions, and mutations in near-gene 5' and near-gene 3' (Raghav and Sharma 2013). Of the 79 nonsynonymous missense mutations 29 are identified as native protein conformation changing (Raghav and Sharma 2013). Such protein conformation altering mutations in the *ERBB3* gene are most common in gastric and colon cancers (12% and 11% of all cancer cases of the named type, respectively.) (Jaiswal et al. 2013). 2–4% of breast cancers, on the other hand, have been reported to contain nonsynonymous point mutations in the *ERBB3* gene. The significance of these mutations to the onset and development of cancer has not been evaluated (Jaiswal et al. 2013).

2.5.2. Point mutations in the HER-3 receptor

A few recurrent point mutations in the HER-3 protein have been found. Most of these mutations are located in the extracellular region of HER-3, but some are also located in the intracellular domain (Jaiswal et al. 2013). Nine of the mutation positions (V104, A232, P262, G284, D297, G325, T355, S846 and E928; seven in the extracellular region and two in the kinase domain) have been shown to exist in multiple samples, and therefore they are identified as mutational hot spots (Jaiswal et al. 2013). Two of the nine hot spot mutations (valine 104 to methionine and glutamate 928 to glutamine) are present in breast cancer while the other seven are primarily found in colon and gastric cancers (Jaiswal et al. 2013). In each hot spot position, the original amino acid is substituted to a same new amino acid in most cases. This indicates that the changes could be functionally relevant and that there could be some driving force for the mutations (Jaiswal et al. 2013).

One of the seven mutational hot spots found in the extracellular domain of HER-3 (valine 104 to methionine) is in the subdomain I, five (alanine 232 to valine, phenylalanine 262 to histidine/serine, glycine 284 to arginine, aspartate 297 to tyrosine, and glycine 325 to argine) are in the subdomain II, and one (threonine 355 to alanine/isoleucine) is in the subdomain III (Jaiswal et al. 2013). The mutations at V104, A232 and G284 are located in the interface of subdomains I and II, and it has been suggested that they might affect the structure of the disulfide-containing modules in domain II (Jaiswal et al. 2013). This might lead to enhanced,

or possibly decreased, dimerization activity. P262 is thought to participate in the interactions between domains II and IV which keep the receptor in closed conformation. Therefore it is possible that HER-3 receptors having a mutated amino acid residue at position 262 are more easily converted to open conformation than are wild type receptors (Jaiswal et al. 2013). D297 is located near the dimerization arm of subdomain II and it has an important role in heterodimerization (Jaiswal et al. 2013). Mutations of this residue might hence increase the number of HER-2/HER-3 dimers. G325 and T355 are located in the border region between subdomain II and III of the extracellular domain (Jaiswal et al. 2013). Rather large conformational changes occur in the border region, and mutations of residues 325 and 355 might have an impact on these transitions.

The two mutational hot spots found in the intracellular segment of HER-3 (serine 846 to isoleucine and glutamate 928 to glycine) are both located in the kinase domain. S846 is located near the path taken by the to be phosphorylated C-terminal tail in dimeric kinase structures (Jaiswal et al. 2013). E928 is also believed to be involved in heterodimer formation. Significance of the mutations in this residue, however, is not known (Jaiswal et al. 2013).

Several of the above mentioned hot spot mutations of HER-3 receptor promote oncogenic signaling in cancer cells if active HER-2 receptors are present (Jaiswal et al. 2013). For example, HER-3 receptors containing mutations at V104, A232, and P262 can form dimers with HER-2 and activate downstream signaling pathways without the HER-3 ligands NRG-1 and NRG-2 (Jaiswal et al. 2013). However, the activity of HER-3 receptors containing these mutations do increase when NRG-1 or NRG-2 is added, and therefore it is believed that these mutations do not necessarily force the receptor to open conformation but only rather make the closed conformation less favourable than in wild-type receptors (Jaiswal et al. 2013).

In addition to the hot spot mutations, several other important point mutations in the HER-3 receptor have been reported, most of them being located in domains I, II and III of the extracellular segment. Glycosylation site Asn414, for example, has been found to be critical to regulation of HER-3 function: if the asparagine residue is mutated into glutamine, the receptor can dimerize with HER-2 in the absence of ligand and thus the cancerous properties of a cell are enhanced (Sithanandam and Anderson 2008). Other non hot spot mutations which have been reported to disrupt the conformation of HER-3 receptor include V89M, V105G, C290Y, T389K, I418N, R669C, I744T, and A1131T (Jaiswal et al. 2013, Raghav and Sharma 2013). All these mutations are considered as potentially disease causing (Raghav and Sharma 2013).

2.6. Dimerization of HER family receptors

2.6.1. Overview of the dimerization of HER family receptors

All members of the HER family form hetero- and homodimers with each other in order to convert extracellular signals into intracellular signals. Before receptors can dimerize, they must bind an associated ligand that changes the conformation from closed to open (Harbeck et al. 2013). HER-2 is an exception as it continuously exists in the open conformation and does not bind any known ligand (Harbeck et al. 2013). When two receptors that are in open conformation have dimerized, they can be autophosphorylated (Burgess et al. 2003, Zhang et al. 2006). Phosphorylation is followed by receptor internalization and further downstream signaling (Wang et al. 2010, Harbeck et al. 2013).

2.6.2. HER-2/HER-3 dimer

Of all the dimers formed by the members of the HER family, HER-2/HER-3 heterodimers are the most potent activators of downstream signaling (Amin et al. 2010). It has been suggested that cell transformation and breast carcinoma progression might be triggered by HER-2/HER-3 heterodimers: HER-3 is considered as an irreplaceable dimerization partner of HER-2, and it is thought to be required for proliferation of HER-2 positive breast cancer cells (Holbro et al. 2003, Choi et al. 2012, Green et al. 2014). It is noteworthy, though, that cancer cells with no or few HER-3 molecules are able to maintain similar levels of phosphorylated HER-2 receptors as are cells with high levels of HER-3 (Holbro et al. 2003). However, the cells containing only a few HER-3 receptors are proliferatively blocked, which confirms the great importance of HER-3 in breast cancer (especially in HER-2 positive subtype) development (Holbro et al. 2003). The antiproliferative properties resulting from the loss of HER-3 could not be replaced by HER-1 or HER-4 proteins (Holbro et al. 2003).

As reviewed in Holbro et al. (2003), the importance of cooperative actions of HER-2 and HER-3 in the development of breast cancer has been further demonstrated in several studies which have shown that blocking of HER-2 also decreases signaling ability of HER-3 (Lane et al. 2000, Neve et al. 2000, Basso et al. 2002, Motoyama et al. 2002). In addition to the *in vitro* models, an *in vivo* study of transgenic mammary tumors showed elevated levels of tyrosine phosphorylated HER-3 in HER-2 positive tumors, while the levels of phosphorylated HER-1 or HER-4 were not increased (Holbro et al. 2003).

All the above mentioned observations suggest that HER-3 is the most important and biologically relevant partner of HER-2 in HER-2 positive breast cancers: HER-3 as a

dimerization partner to HER-2 is believed to be required to fulfill the full signaling potential of HER-2 positive breast cancer cells (Holbro et al. 2003). In addition to their potency, HER-2/HER-3 dimers are common in breast cancer, especially in HER-2 positive subtype. According to one study, HER-2/HER-3 dimers are detectable in 13.1% of all breast cancers and in 73% of HER-2 positive tumors (Green et al. 2014).

Dimerization of HER-2 and HER-3 promotes cell proliferation and inhibits apoptosis mainly by enhancing phosphatidylinositide 3-kinase (PI3K)/Akt signaling pathway (Kruser and Wheeler 2010, Choi et al. 2012). The PI3K/Akt pathway is a classic survival pathway that is activated in many different cancers (LoPiccolo et al. 2008). The pathway is iniatiated when cell membrane located PI3K is activated. Members of the HER family, integrings or G-protein-coupled receptors, for example, can activate PI3K (LoPiccolo et al. 2008). Activated PI3K catalyzes phosphorylation of phosphoinositides generating phosphatidylinositol-3,4,5-triphosphate (PI(3,4,5)P3) and phosphatidylinositol-3,4-bisphosphate (PI(3,4)P2) (LoPiccolo et al. 2008). Phosphorylated phosphoinositides bind to Akt, a serine/threonine kinase, and cause it to move to cell membrane where it is activated. Akt is known to exist as three isoforms, Akt1, Akt2 and Akt3. Akt isoforms are structurally homologous (LoPiccolo et al. 2008), and they are activated similarly (not explained here in detail). All Akt isoforms phosphorylate a consensus sequence RXRXX(S/T) present in numerous proteins. Because the consensus sequence is rather common, several Akt substrates such as FoxO, BAD, IKK, and most importantly mTOR have been identified (LoPiccolo et al. 2008). These substrates control important cellular mechanisms like cell cycle progression, transcription, and translation (LoPiccolo et al. 2008).

HER-2/HER-3 heterodimers enhance PI3K/Akt pathway through direct HER-3 binding to PI3K. HER-3 contains six tyrosine-binding sections, and thus it has the highest affinity for PI3K among all of the receptors of HER family; no other member of the HER family is capable of activating the PI3K/Akt pathway as strongly as HER-3 (Holbro et al. 2003, Stern 2008, Green et al. 2014).

Regardless of the significance of HER-3 in the activation of PI3K/Akt pathway, it is noteworthy that HER-3 alone or as a homodimer is not able to activate the pathway (Green et al. 2014). Additionally, despite the indisputable importance of the PI3K/Akt pathway in breast cancer development, HER-2/HER-3 dimers can also activate other downstream signaling pathways such as PLCγ/PKC, Jak/Stat, and MEK/MAPK (not described here in detail) (Ma et al. 2014).

2.6.3. HER-2/HER-3 dimer targered therapy

Due to the fact that HER-2/HER-3 dimers are a rather new target for drug development, only one agent, pertuzumab, directly inhibiting the dimer formation is currently in clinical testing. Pertuzumab is a humanized monoclonal IgG1 antibody that binds to the extracellular dimerization domain (subdomain II) of HER-2 receptor, efficiently blocking ligand-dependent heterodimerization with other members of the HER family, especially with HER-3 (Baselga J 2010, Harbeck N et al. 2013, Malenfant et al. 2014). This blocking leads to the inhibition of downstream signaling pathways and consequently to increased rates of apoptosis and cell growth suspension (Malenfant et al. 2014).

2.6.4. Combination of pertuzumab and trastuzumab in the treatment of breast cancer

Pertuzumab binds to the extracellular domain of HER-2 in a similar fashion as trastuzumab, another humanized monoclonal antibody used to treat HER-2 positive breast cancer. Trastuzumab, like pertuzumab, activates antibody-dependent cellular cytotoxicity (Zagouri et al. 2013). Pertuzumab and trastuzumab, however, bind to different epitopes of HER-2: pertuzumab binds to the center of the subdomain II of HER-2 extracellular domain, while trastuzumab binds to the subdomain IV of the extracellular domain (Harbeck N et al. 2013). Trastuzumab inhibits ligand-independent HER-2 signaling, pertuzumab prevents HER-2/HER-3 dimerization, and together they induce apoptosis both *in vitro* and *in vivo* (Lee-Hoeflich et al. 2008, Scheuer et al. 2009, Harbeck N et al. 2013). Results from several studies have suggested that this kind of treatment of HER-2 positive breast cancer with two monoclonal antibodies which have different mechanisms of action and distinct binding sites results in a more complete blockage of downstream signaling pathways when compared to a treatment with either of the antibodies alone (Ma et al. 2014). It is noteworthy, though, that in HER-2 negative breast cancers a combination treatment with trastuzumab and pertuzumab is not more effective than a treatment with pertuzumab alone (Zagouri et al. 2013). Complementary mechanisms of action of trastuzumab and pertuzumab are shown in figure 6.

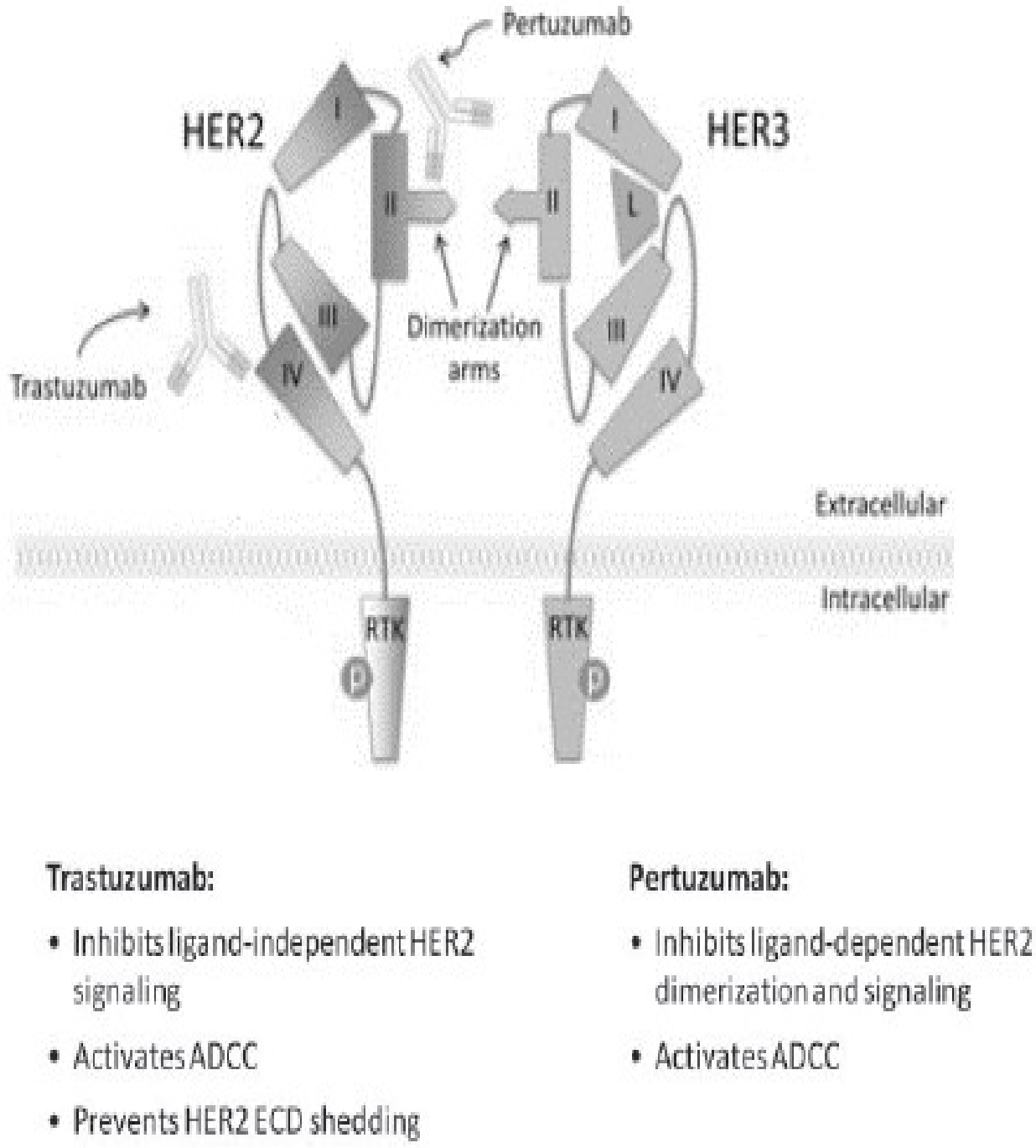

Figure 6. Compelentary mechanisms of action of trastuzumab and pertuzumab. Image adapted from Harbeck et al., (2013) and Malenfant et al., (2014).

2.7. Theoretical backgroung of the methods used in this study

2.7.1. Proximity ligation assay

In proximity ligation assay (PLA) two interacting, closely located (maximum distance 30 nm) proteins can be detected and quantitated (Söderberg et al. 2006). PLA is based on the simultaneous and proximate recognition of target proteins by an affinity probe pair. (Fredriksson et al. 2002). At the beginning of the assay, two primary antibodies are incubated

with samples such as fixed cells. After the primary antibodies have bound to their target proteins, secondary antibodies conjugated with oligonucleotides (around 40 nucleotides long each) are added. Two secondary antibodies, each recognizing only one primary antibody, are used. Linear connector oligonucleotides are added after the secondary antibodies and, if the target proteins are sufficiently close to each other, a circular structure is formed and ligated by DNA ligase (Söderberg et al. 2006). Nucleotides, fluorescently labeled oligonucleotides and DNA polymerase (phi29) are then added. The oligonucleotide chain of one of the secondary antibodies functions as a primer for rolling circle amplification reaction that uses the previously formed circle as a template (Fredriksson et al. 2002). The circle is duplicated up to 1000 times, and all the generated copies of the circle are covalently linked to each other. The fluorescently labeled oligonucleotides then hybridize with the nucleotides in the product molecule generating a fluorescent signal visible by fluorescent microscopy (Fredriksson et al. 2002). Theoretically, each dimer produces a single dot and therefore it is possible to count the total amount of dimers in a single cell (Fredriksson et al. 2002).

A simple series of pictures showing the principle of the assay is shown in figure 7. It should be noted, though, that there are also alternative protocols for PLA, but they are not presented here as they were not used in this study (Anonym 2010).

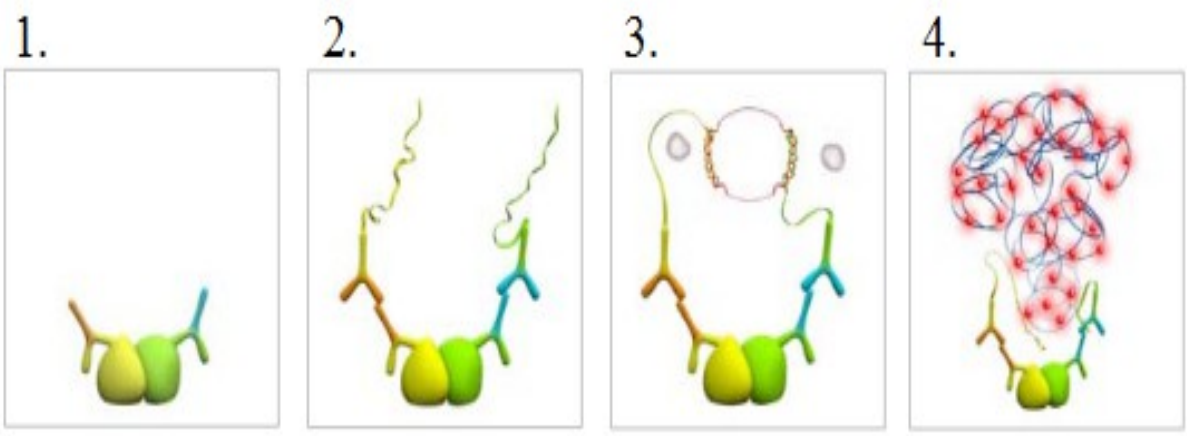

Figure 7. A simple series of pictures showing the principle of PLA. In (1.), primary antibodies have bound to the target proteins. In (2.), secondary antibodies to which oligonucleotide chains have been attached have bound to the primary antibodies. In (3.), linear connector oligonucleatides (red bands) have been added, and a circular structure has been formed and ligated by DNA ligase (white). In (4.), DNA polymerase (not shown) has multiplied the circle, and fluorescently labeled oligonucleotides (red dots) have hybridized with the nucleotides in the product molecule. Adapted from anonym (2010).

2.7.2. *In vitro* drug sensitivity assay

In vitro drug sensitivity assay was performed using a commercial kit. According to the manufacturer, "membrane-permeant calcein AM is cleaved by esterases in live cells to yield the cytoplasmic green fluorescence" (Anonym 2014). Membrane-impermeant ethidium

homodimer-1, on the other hand, labels nucleic acids of dead (membrane-compromised) cells with red fluorescence (Anonym 2014).

3. Main goals of the thesis

The main goals of this thesis were:

1. To quantitate the amount of HER-2/HER-3 dimers in different HER-2 positive cancer cell lines using proximity ligation assay and to test how pertuzumab affects the dimerization.

2. To study the drug sensitivities of different HER-2 positive cancer cell lines to pertuzumab and trastuzumab, and to compare the obtained results to the results of proximity ligation assay experiments. The previous observation that a combination treatment with pertuzumab and trastuzumab leads to a more complete blockage of downstream signaling pathways than either of these drugs alone was also further evaluated.

3. To evaluate the abundance and the possible importance of HER-3 point mutations in breast cancer, especially in the HER-2 positive subtype, using *in silico* methods. Addtionally, the relationship between HER-3 point mutations and pertuzumab sensitivity was tested *in vitro*.

4. Materials and methods

4.1 Cell lines

The human breast cancer cell lines BT-474, HCC1419, MDA-MB-361, MDA-MB-453, MCF-7, SK-BR-3, UACC-893, and ZR-75-30 were obtained from the American Type Tissue Culture Collection, and the cell line EFM-192A was obtained from the German Resource Center for Biological Material. The human oesophagus/gastric carcinoma cell line OE19 was obtained from the European Collection of Cell Cultures. The human breast cancer cell line JIMT-1 has been established in the laboratory of Cancer Biology, University of Tampere, Finland (Tanner et al. 2004). Cells were grown under conditions recommended for each line.

4.2. Proximity ligation assay

4.2.1 Basic protocol

Proximity ligation assay (PLA) was performed using Duolink In Situ Fluorescense kit (Olink Biosciences, Uppsala, Sweden). Before starting the PLA protocol cells were trypsinated and deposited on 8 well Nunc Lab-Tek II Champer slides (Thermo Fisher Scientific Inc., Waltham, MA, USA). Cells were allowed to attach and grow to ~80% confluency before they were fixed (10 minutes in 10% neutral-buffered formalin) and permeabilized (2,5 minutes in 0,25 % Triton-X 100). After fixation and permeabilization cells were blocked with Duolink blocking solution for 30 minutes in a pre-heated humidity chamber at 37°C. After the blocking step cells were washed with PBS and incubated with two primary antibodies (anti-HER-2 (rabbit mAb, Epitomics EP1045Y, concentration unknown, diluted 1:500) and anti-HER-3 (mouse mAb, Dako DAK-H3-IC, concentration 200 µg/ml, diluted 1:100)) in humidity chamber at 37°C for 60 minutes. Cells were then washed with PBS and incubated with diluted PLA probe solution in humidity chamber at 37°C for 60 minutes. After this cells were incubated 30 minutes with ligation solution followed by a wash step and 100 minutes incubation with amplification solution, both incubations taking place at 37°C in a humidity chamber. Finally, slides were dried and mounted with Vectashield mounting medium with DAPI.

An Axio Apotome microscope (Carl Zeiss Microscopy) equipped with an AxioCam Mrm camera using a 63x oil immersion objective (Zeiss Plan-Neofluar) was used for PLA microscopy. Depending on cell line, 2–4 fields of view were used and 52–86 individual cells were imaged. 15 stack images with a slice distance of 1.000 µm were taken from each field of view. Exposure time of 400 ms was used for the red dye and exposure time of 10 ms was used

for DAPI.

Dimers were visualized as distinct fluorescent dots (diameter 0.5-1.5 μm). ImageJ software was used to quantitate the dots in image focus stacks. Every second image of each image focus stack was used, and thus the distance between each analyzed image in each stack was 2 μm. The images were converted to 8-bit images and thresholded appropriately (pixel intensity values between 30 and 255 were used). After thresholding, analyze particles -tool of imageJ was used to calculate total amount of dimers in image stacks. The amount of dimers in a few images was manually counted to evaluate the automated protocol.

4.2.2 PLA for pertuzumab-sensitized cells

After the basic proximity ligation assay protocol had been optimized, PLA was performed for pertuzumab-sensitized cells. The cells were first deposited on 8-well chamber slides and allowed to attach overnight. After the cells had attached, 20 μg/ml pertuzumab was added into the non-control wells and the cells were incubated for 72 hours. A PLA protocol identical to the protocol described in section 4.2.1 was then performed.

4.3. *In vitro* drug sensitivity assay

The effects of trastuzumab, pertuzumab, and the combination of these drugs on the growth of HER-2 overexpressing cells were examined by LIVE/DEAD Viability/Cytotoxicity Kit (Life Technologies, Carlsbad, CA, USA). HER-2 and HER-3 negative MCF-7 and MDA-MB-231 cell lines were used as negative controls. Cells were first trypsinized and plated at suitable densities (3000–5000 cells per well) in 96-well tissue culture plates and they were allowed to attach overnight before drug addition. The effects of both trastuzumab and pertuzumab were tested at a concentration of 20 μg/ml. The number of viable cells was determined at 72 h after drug addition.

EVOS FL Auto Cell Imaging System (Life Technologies) with a 10x dry (air) objective was used to image the cells. Four images from each well containing a sample were taken manually.

In most of the cell lines, the living cells were too confluent to be counted separately. Because of this, the total amount of cells in each image had to be calculated using an indirect method. First, the surface area of living cells in every sample was determined using imageJ. Then the total amount of ZR-75-30 cells were manually counted and the average surface area of a single cell was determined. This average surface area was then used to derive the total amount

of living cell in every cell line. ZR-75-30 cells were used as reference because they were the least confluent of all cell lines and thus relatively easy to count. Dead cells were counted manually because there were relatively few of them in each cell line. When the amount of both dead cells and living cells had been obtained, a ratio of the amount of living cells divided by the number of dead cells was calculated for each sample. The ratio of each drug-sensitized sample was then divided by the ratio of corresponding control sample to obtain the percentage difference between the drug-sensitized samples and the control sample. The smaller the percentage, the bigger effect the drug had on the cell line in question.

4.4. *In silico* mutational analysis

Frequency of point mutations in the *ERBB3* gene in breast cancer tissue samples was determined using the cBioPortal (Cerami et al. 2012, Gao et al. 2013). The portal contained four distinct breast cancer datasets generated by British Columbia Cancer Research Centre, by the Broad Institute, and by the Sanger Institute (Banerji et al. 2012, Shah et al. 2012, Stephens et al. 2012). Additionally, a breast cancer dataset from TCGA (The Cancer Genome Atlas) was included in the *in silico* mutational analysis.

COSMIC database was used to determine whether or not the cell lines used in this study contain mutations in the *ERBB3* gene (Forbes et al. 2008, Forbes et al. 2011).

5. Results

5.1. Amount of HER-2/HER-3 dimers in breast cancer cell lines

Proximity ligation assay was used to determine the amount of HER-2/HER-3 dimers in one gastric carcinoma cell line and in 12 breast cancer cell lines. Results are shown in figure 8. Two (MCF-7 and MDA-MB-231) of the total 13 different cancer cell lines were known to be HER-2 negative and they were used as negative controls in this part of the study. The results were expected, as the negative samples had notably less dimers than most other samples.

To confirm that the fluorescent dots visualized really were HER-2/HER-3 dimers, HER-2/HER-3 dimerization inhibitor pertuzumab was incubated 72 hours with cells, after which a standard proximity ligation assay protocol was performed. Percentages of dimers in pertuzumab sensitized samples compared to control samples are shown in figure 9. Results show that pertuzumab has at least a moderate effect on the amount of dimers of five (BT-474, EFM-192A, HCC1419, MDA-MB-231, and UACC-812) of the ten tested cell lines. Two cell lines (SK-BR-3 and OE19) show a little reduction in the amount of dimers, while three cell lines (JIMT-1, MDA-MB-453, and ZR-75-30) are unaffected.

Example PLA images of different cell lines are shown in appendix 1, figure S1. Each image shown have been averaged from all the stack images obtained from a single field of view. Therefore, not all dimers are visible in the images.

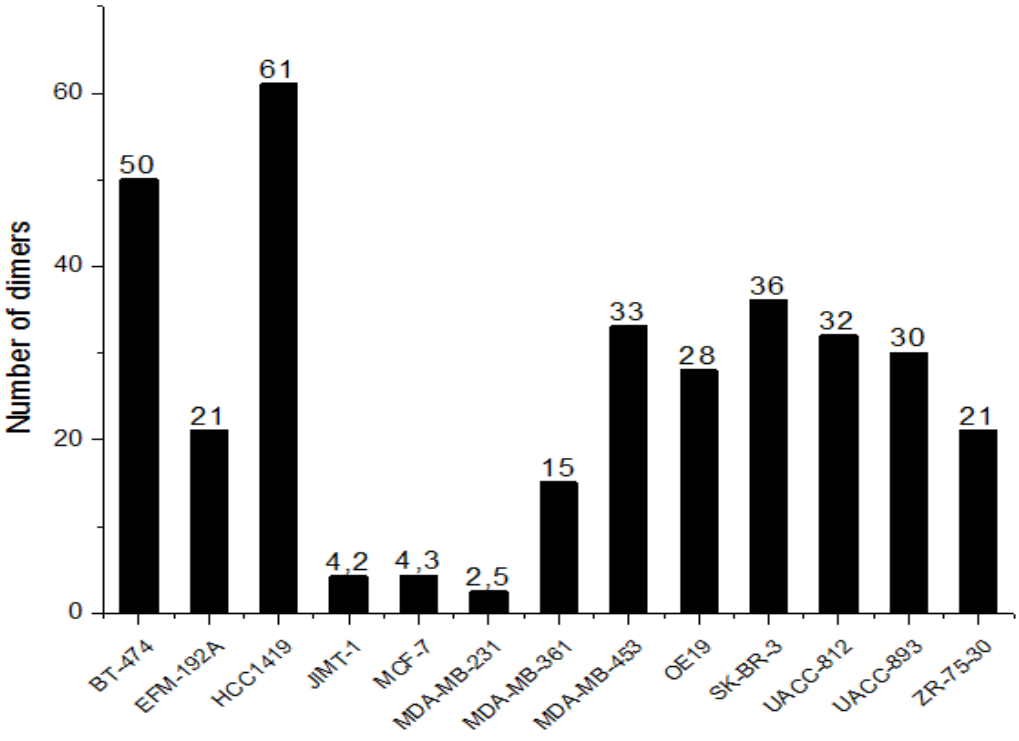

Figure 8. Amount of dimers per cell in different cancer cell lines.

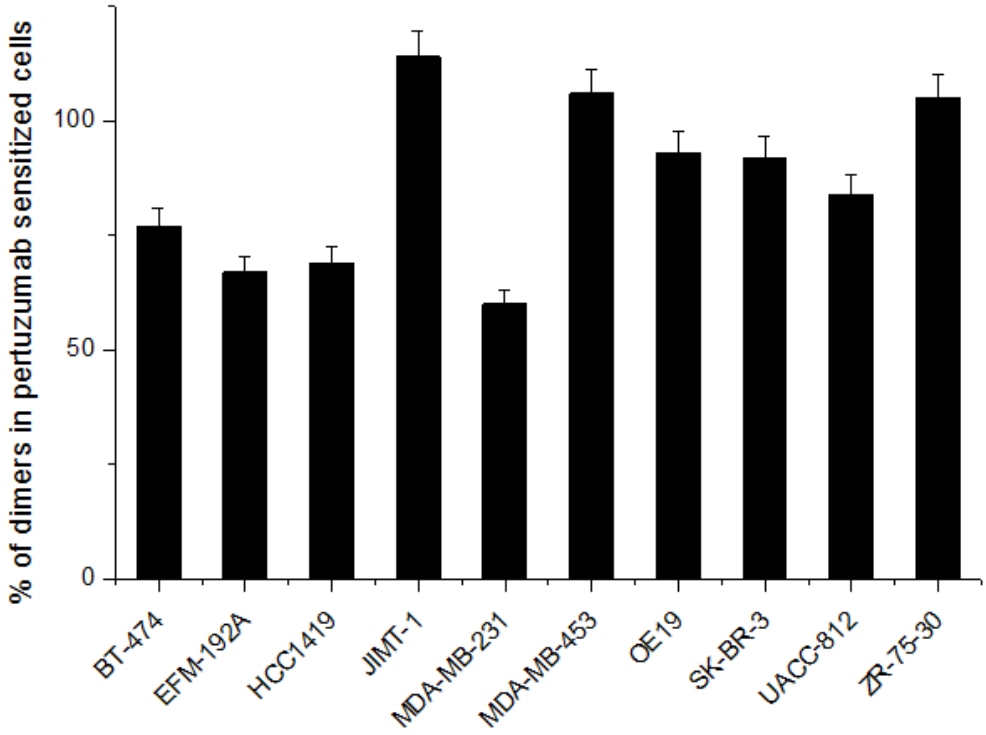

Figure 9. Amount of dimers in pertuzumab sensitized cells. 100% corresponds the amount of dimers in contrtol samples.

5.2. Drug sensitivity in HER-2 positive cell lines

A commercial LIVE/DEAD Viability/Cytotoxicity Kit (Life Technologies) was used to determine the sensitivity of 12 breast cancer and a single gastric cancer cell lines to pertuzumab, trastuzumab, and the combination of these drugs. Results of the drug sensitivity assay are shown in figure 10.

Trastuzumab alone showed a significant (<90% proportion of living cells/dead cells in a drug-sensitized sample compared to the corresponding control sample) effect in five of the twelve cell lines. Pertuzumab alone showed a significant effect in seven of the thirteen cell lines. Combination of both of the above mentioned drugs showed significant effect in ten of the thirteen cell lines. Two (MCF-7 and MDA-MB-231) of the three cell lines which did not show significant reaction to trastuzumab, pertuzumab, or the combination of these drugs were the negative control cell lines of this experiment, and therefore the observed results were expected. The third line (JIMT-1) that did not show significant response to either of the drugs has previously been shown to be trastuzumab resistant, and this experiment indicates that it is also resistant to pertuzumab (Köninki et al. 2010). This was expected as JIMT-1 cells contain only a few dimers as demonstrated in figure 8.

Even though most of the results seem reasonable, a few of them are surprising. Three of the cell lines showing high quantity of HER-2/HER-3 dimers (BT-474, ZR-75-30 and SK-BR-3) seem to be unaffected by pertuzumab. Particularly the insensitivity of BT-474 cells to pertuzumab is surprising, as the drug significantly decreased the amount of dimers in this cell line (figure 9). Additionally, MDA-MB-361 cells which show the strongest reaction to pertuzumab have relatively few dimers when compared to for example HCC1419 cells.

Example images of different cell lines after the addition of the LIVE/DEAD reagent are shown in appendix 1, Figure S2.

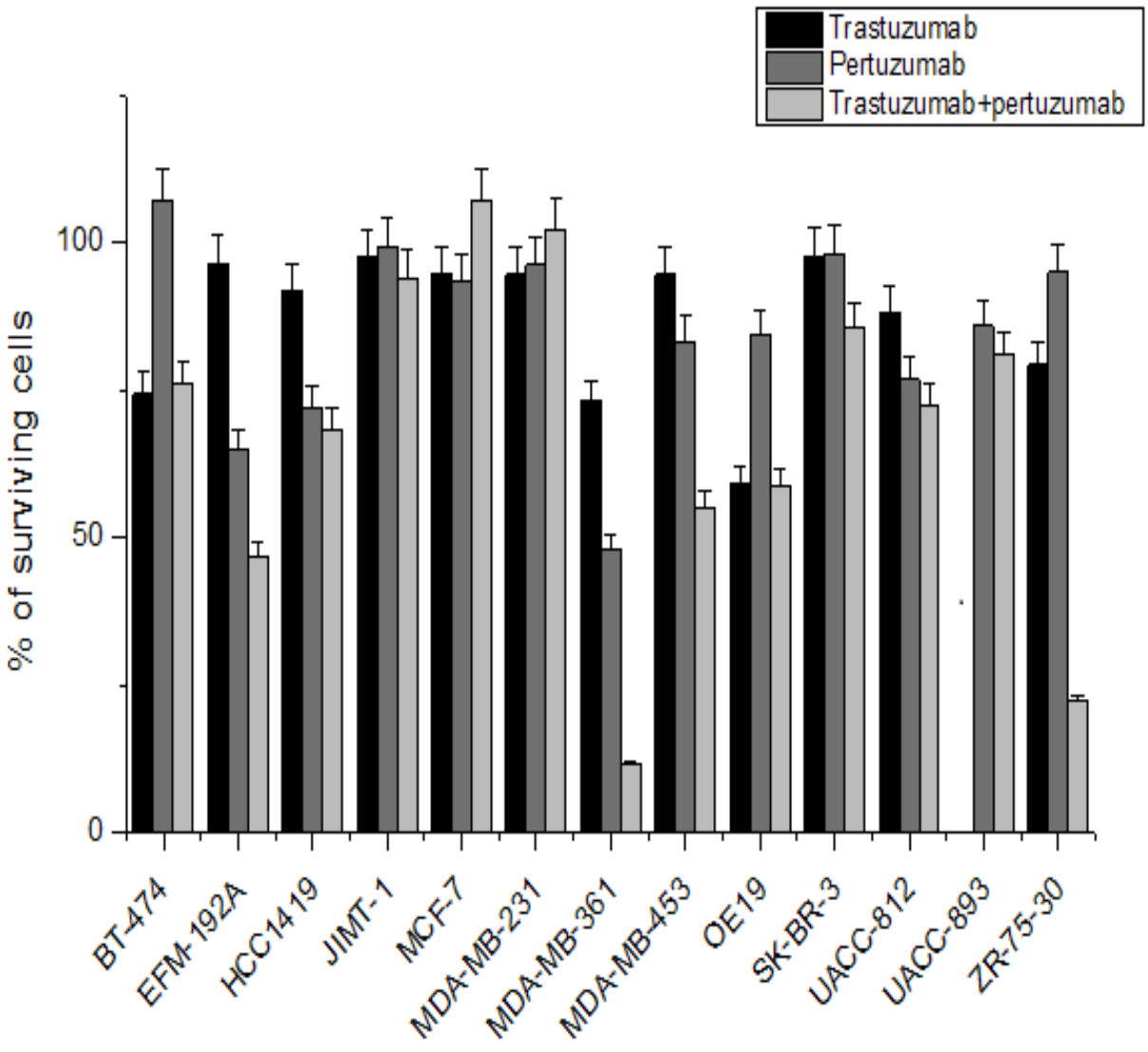

Figure 10. Results of the drug sensitivity assay. Percentage of the proportion of living cells/dead cells in drug-sensitized samples are shown. 100% corresponds the proportion of living cells/dead cells in contrtol samples. The effect of both trastuzumab and pertuzumab was tested at a concentration of 20 µg/ml.

5.3. HER-3 point mutations in breast cancer

The cBioPortal contained data from a total of 1230 breast cancer tumor samples when this *in silico* study was performed. Of these samples 20 contained a nonsynonymous point mutation in the *ERBB3* gene (1.62% of all samples). Two mutations, leading to amino acid changes T355I and D279Y, occured in two different samples, while 16 mutations occured only in one sample.

GISTIC 2.0 tool (Mermel et al. 2011) had been previously used to determine the amount of HER-2 amplified samples among the TCGA samples. 119 (12.4%) of the 962 TCGA samples were HER-2 amplified. The percentage is a little lower than expected, but it still seems reasonable considering that the results obtained by the GISTIC tool are only putative. Four of the HER-2 amplified samples (3.36% of all HER-2 amplified samples) and 12 of the non-HER-2 amplified samples (1.42% of all non-HER-2 amplified samples) contained nonsynonymous point mutations in the *ERBB3* gene.

According to the COSMIC database, none of the studied cell lines contain point mutations in HER-3. SK-BR-3 cell line is not listed in the COSMIC database, but according to the CCLE these cells contain a nonsynonymous point mutation, leading to amino acid change E952Q in the *ERBB3* gene. The mutation is located in the kinase domain of HER-3 and it is not identified as a hot spot mutation

6. Discussion

6.1. Proximity ligation assay

Proximity ligation assay can be considered to be a rather reliable method in the quantification of HER-2/HER-3 dimers. As expected, the amount of dimers in HER-2 negative cell lines are considerably smaller than in HER-2 positive cell lines, and addition of pertuzumab decreases the amount of dimers in most cell lines. Additionally, negative control samples (only one primary antibody used) show hardly any dimers, whereas the sample in which two anti-HER-2 antibodies were used shows signal so strong that it is impossible to calculate single dots.

Flow cytometry results (table 1) obtained by Satu Luhtala (Cancer Biology Group, BioMediTech, University of Tampere, Finland) correlate well with proximity ligation assay results presented earlier (figure 8 and figure 9), further confirming the accuracy of PLA. Cell lines which have only few dimers (JIMT-1 and MDA-MB-231) show smaller fluorescent readings in HER-2 and especially in HER-3 when compared to other cell lines. HER-3 seems to be the limiting factor of dimer formation as the fluorescent reading of HER-3 is much lower than the reading of HER-2. Different antibodies for HER-2 and HER-3, on the other hand, were naturally used, and thus smaller fluorescence values of HER-3 does not necessarily mean that significantly fewer HER-3 receptors are present in cells. Additionally, HER-2 fluorescence values were measured from saponin-permeabilized cells while the HER-3 fluorescence values were measured from non-permeabilized cells.

Table 1. Flow cytometry results. Fluorescent values measured by the BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA) are shown. The total amount of receptors cannot be deducted from the results.

Cell line	HER-3 fluorescence	HER-2 fluorescence
BT-474	292 110	8 236 960
EFM-192A	-	7 288 670
HCC1419	178 540	10 284 300
JIMT-1	43 650	1 099 780
MCF-7	-	322 210
MDA-MB-231	27 150	170 200
MDA-MB-361	-	2 128 850
MDA-MB-453	170 920	1 490 260
OE19	-	6 679 420
SK-BR-3	192 130	3 964 280
UACC-812	-	9 489 330
UACC-893	-	11 913 480

Even though most of the PLA results seem reasonable, some of them are surprising. First, two of the cell lines having many dimers (MDA-MB-453 and ZR-75-30) seem to be unaffected by pertuzumab. Second, MDA-MB-231 cell lines with very few dimers seem to be strongly affected by pertuzumab. In this case, however, the percentage in the figure 9 is misleading. This is due to the fact that in a sample with few dimers, a small decrease in the amount of dimers can lead to large decrease in percentage. In the case of MDA-MB-231 cells the average amount of dimers per cell decreased only by one, which corresponds 40 percent decrease.

Even though PLA is an easy technique to perform, the image analysis proved to be relatively difficult: choosing the right threshold values for the images, defining the size and shape of a single dimer, and choosing the optimum distance between the stack images were no simple tasks. The diameter of a single dimer varied a lot, from 0,5 µm to 1,5 µm, and this led to problems when choosing the image analysis settings. Additionally, when cell lines which contained many dimers were analyzed, it was hard to say whether a single dot was a single dimer or if two or more dimers had merged into a single dot. Therefore the results of cell lines containing a lot of dimers (HCC1419, BT-474, SK-BR-3, MDA-MB-453, UACC-893, UACC-812, and OE19) might be too low when compared to cell lines with only a few dimers, such as JIMT-1.

6.2. Drug sensitivity assay

The results obtained from the drug sensitivity assay are reasonable. Pertuzumab and trastuzumab don't seem to have effect on HER-2 negative cell lines, which is expected. Additionally, in the majority of the studied cell lines the combination of these two drugs had stronger effect on cell survival than either of the drugs alone. Obtained results also correlate well with previous studies. In a study by Barok et al. (2011), for example, the three most sensitive cell lines (MDA-MB-361, ZR-75-30 and BT-474) to trastuzumab were also among the most sensitive cell lines to trastuzumab in this study. The results of this previous trastuzumab drug sensitizing experiment are shown in figure 11.

Still, even though the results seem to be rather reasonable, the method used in the drug sensitivity assay cannot be considered optimal. This is mainly due to the fact that only relatively few dead cells were present in most samples, and the amount of living cells were usually a few dozen times greater than the amount of dead cells. Thus, only a little change in the amount of dead cells could have had a significant impact on the obtained results. This

could explain the observation that three of the cell lines showing high quantity of HER-2/HER-3 dimers (BT-474, ZR-75-30 and SK-BR-3) seem to be unaffected by pertuzumab.

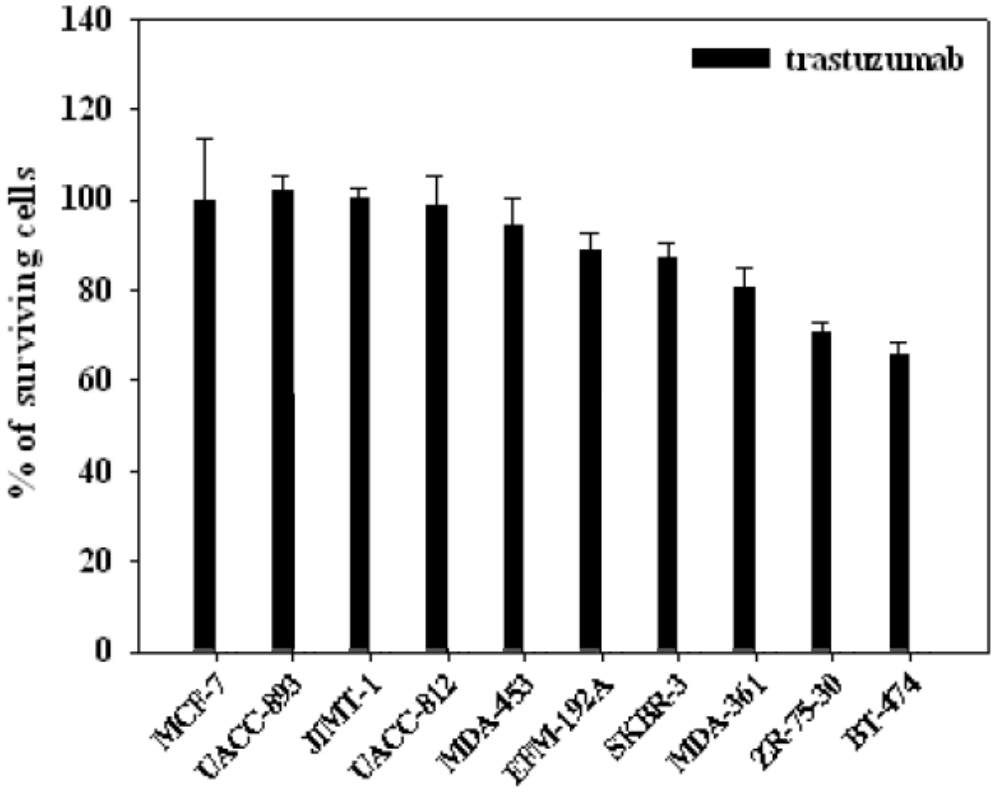

Figure 11. Percentage of surviving cells after 72 hours incubation with trastuzumab (10 µg/ml). Figure adapted from Barok et al., (2011).

6.3. *In silico* mutation analysis

Point mutations in the *ERBB3* gene are not very common in breast cancer (1.62% of the 1230 samples studied contained a nonsynonymous missense mutation). Five (E928G, D297Y, V104L, T355I, and G284R) of the 18 different HER-3 point mutations found in the breast cancer samples are located in mutational hot spot sites (Jaiswal et al. 2013). One of these mutations (V104L) is located in the subdomain I of extracellular domain, two (D297Y and G284R) are located in the subdomain II of the extracellular domain, one (T355I) is located in the subdomain III of the extracellular domain, and one (E928G) is located in the kinase domain. The fact that majority of these mutations are located in the extracellular domain indicates that most of the HER-3 point mutations affect ligand binding or dimerization. D297, for example, is located near the dimerization arm of subdomain II and it has an important role in heterodimerization (Jaiswal et al. 2013). However, further and more precise structural analysis of the HER-3 receptor is needed to evaluate the full impact of these mutations to the function of HER-3.

An itriguing fact is that in breast cancers in which the *ERBB2* gene is amplified, the frequency of point mutations in the *ERBB3* gene is more than two times higher than in other subtypes of breast cancer. The total amount of mutations in all samples (20), though, is so low that it is hard to conclude if this is a signifacnt observation or just a coincidence. It is therefore important to obtain more sequencing data from breast cancer samples in the future to better evaluate the abundance and importance of HER-3 mutations.

The only cell line used in this study that contains a nonsynonymous point mutation in the *ERBB3* gene is SK-BR-3. This mutation leads to the amino acid change of glutamic acid (glutamate) 952 to glutamine. The structure of these two amino acids is rather similar (figure 12), so it could be assumed that this mutation does not lead to significant changes in the three dimensional structure of the HER-3 receptor. Detailed structural analysis is required to confirm this assumption.

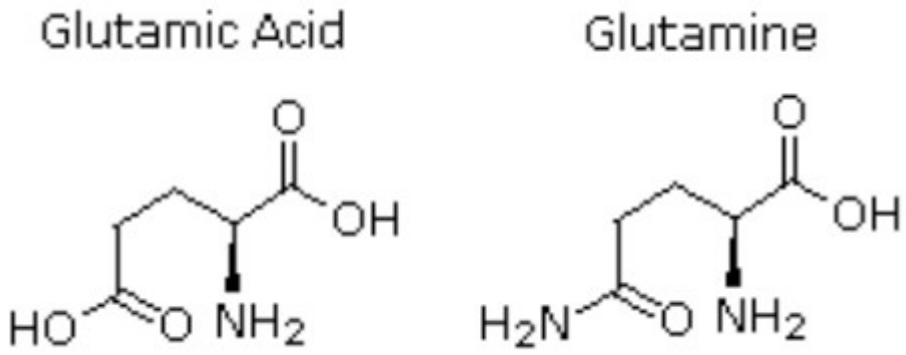

Figure 12. The skeletal formulas of glutamic acid and glutamine. As can be seen, the structure of these amino acids is similar: the only difference between these molecules is that glutamic acid contains a carboxyl group in carbon 5, while glutamine contains an amide group in carbon 5. Adapted from Reusch (2013).

The mutation in SK-BR-3 cells is located in the kinase domain of HER-3, and it might therefore be logical to assume that it cannot affect dimerization. This is, however, not true because the kinase domains of HER family receptors are involved in the dimerization process (Choi et al. 2012).

In the case of SK-BR-3 cells, though, the mutation does not seem to affect the formation of HER-2/HER-3 dimers, as the amount of dimers is high in these cells. Despite the abundance of HER-2/HER-3 dimers, SK-BR-3 cells seem to be resistant to dimerization inhibitor pertuzumab, but it is not possible to say whether this is due to the mutation or not. Because pertuzumab binds to HER-2, a point mutation in the HER-3 receptor cannot affect the binding of this drug to its target. Thus, if the point mutation is responsible for the drug resistance, it

must alter the conformation of HER-3 in a way that it can dimerize with HER-2 receptors to which pertuzumab has bound. Other possibility is that mutated HER-3 receptors can activate the downstream signaling pathways, such as the PI3K/Akt pathway, without the need to dimerize with HER-2. A combination treatment with both trastuzumab and pertuzumab significantly reduced the viability of SK-BR-3 cells even though neither of the drugs alone affected these cells. This implies that if the point mutation in the HER-3 receptor of SK-BR-3 cells is responsible for the obseved insensitivity to pertuzumab, a combination treatment with both trastruzumab and pertuzumab can help overcome this resistance.

In addition to SK-BR-3 cells, HER-2 positive cell lines BT-474 and ZR-75-30 were found to be insensitive to pertuzumab. According to the COSMIC and CCLE databases these two cell lines do not contain nonsynonymous point mutations in their *ERBB3* genes, and therefore the observed drug resistance does not probably stem from HER-3 mutations. It should be noted, though, that both of these projects are unfinished, and it is possible that BT-474 and ZR-75-30 cells do contain point mutations in the *ERBB3* gene altough they are not yet listed in the databases.

6.4. Future perspectives

The high abundance of HER-2/HER-3 heterodimers in HER-2 positive cancers is an important observation. As these dimers activate downstream signaling pathways that lead to cell proliferation and apoptosis inhibition, it is reasonable to use them as drug targets. Furthermore, it has been proposed that HER-2 can't act alone in HER-2 positive cancer cells or tissues, but it needs HER-3 for realizing its full signaling potential (Holbro et al. 2003). Thus, HER-2/HER-3 heterodimers are not a minor aspect of HER-2 positive breast cancer, and the dimers should be considered as an important target for HER-2 positive breast cancer treatment.

Currently only one drug, pertuzumab, directly inhibiting the formation of HER-2/HER-3 dimers is under clinical testing (Harbeck N et al. 2013). Despite previous studies, clinical trials, and the *in vitro* experiments performed in this thesis have shown that pertuzumab can moderately decrease the survival of cancer cells and increase the progression-free survival, the effect is not as good as it could be (Harbeck et al. 2013, Zagouri et al. 2013, Malenfant et al. 2014). For example, Only 7 of the 11 HER-2 positive cancer cell lines used in this thesis reacted significantly to pertuzumab. 10 of the 11 HER-2 positive cell lines, on the other hand, reacted significantly to a combination treatment with both pertuzumab and trastuzumab. The only HER-2 positive cell line not reacting to the combination treatment was JIMT-1, a cell

line previously shown to be resistant to HER2 targeted drug therapy (Tanner et al. 2004). Thus, it is justified to say that pertuzumab should be used in combination with trastuzumab in order to improve the prognosis of patients with HER-2 positive breast cancer. As pertuzumab is the first dimerization inhibitor in clinical trials, it would also be rational to develop new, potentially more efficient HER-2/HER-3 dimerization inhibitors in the future.

Point mutations in the HER-3 receptor could play an important role in the HER-2/HER-3 dimer formation as well as in the onset and development of several different cancer types. One study, for example, found that HER-3 recepor mutants are able to transform colonic and breast epithelial cells in a ligand independent manner (Jaiswal et al. 2013). HER-3 mutants alone, though, are not enough for the transformation, and their oncogenic activity is dependent on the kinase activity of HER-2 (Jaiswal et al. 2013). HER-3 point mutations could also have a significant impact on the effectiveness of HER-3 receptor targeted antibody drugs that are currently under development (Ma el al. 2014). If an amino acid critical for the antibody binding, for example, is mutated, the antibody might not have any effect on its target. It would therefore be important to take these mutations into account when new drugs are developed. A recent study, however, suggests that anti-HER antibodies and small molecule inhibitors can effectively block the signaling mediated by both nonmutated and point mutation containing HER-3 receptors (Jaiswal et al. 2013).

7. Conclusions

The main conclusions from this thesis are:

1. As assumed, HER-2/HER-3 heterodimers are abundant in HER-2 positive cancer cell lines, while HER-2 negative cancer cell lines contain only few dimers.

2. Pertuzumab, a drug inhibiting the formation of HER-2/HER-3 dimers, decreases the amount of dimers in most of the tested cell lines. Pertuzumab alone, though, does not have effect on the survival of four HER-2 positive cell lines (BT-474, JIMT-1, SK-BR-3, and ZR-75-30). Of these cell lines one, SK-BR-3, contains a nonsynonymous missense mutation in the *ERBB3* gene. Whether or not this mutation is responsible for the observed resistance to pertuzumab is unknown.

3. This thesis further confirms that a combination treatment with both pertuzumab and trastuzumab has a greater negative impact on the viability of HER-2 positive cells than either of the drugs alone.

4. Point mutations in HER-3 receptor are not very common in breast cancer, and there are only a few recurring mutations.

5. In breast cancers in which the *ERBB2* gene is amplified, the frequency of point mutations in the *ERBB3* gene is more than two times higher than in other subtypes of breast cancer. This could mean that HER-3 point mutations play an important role in the development of HER-2 positive breast cancer.

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Appendix 1

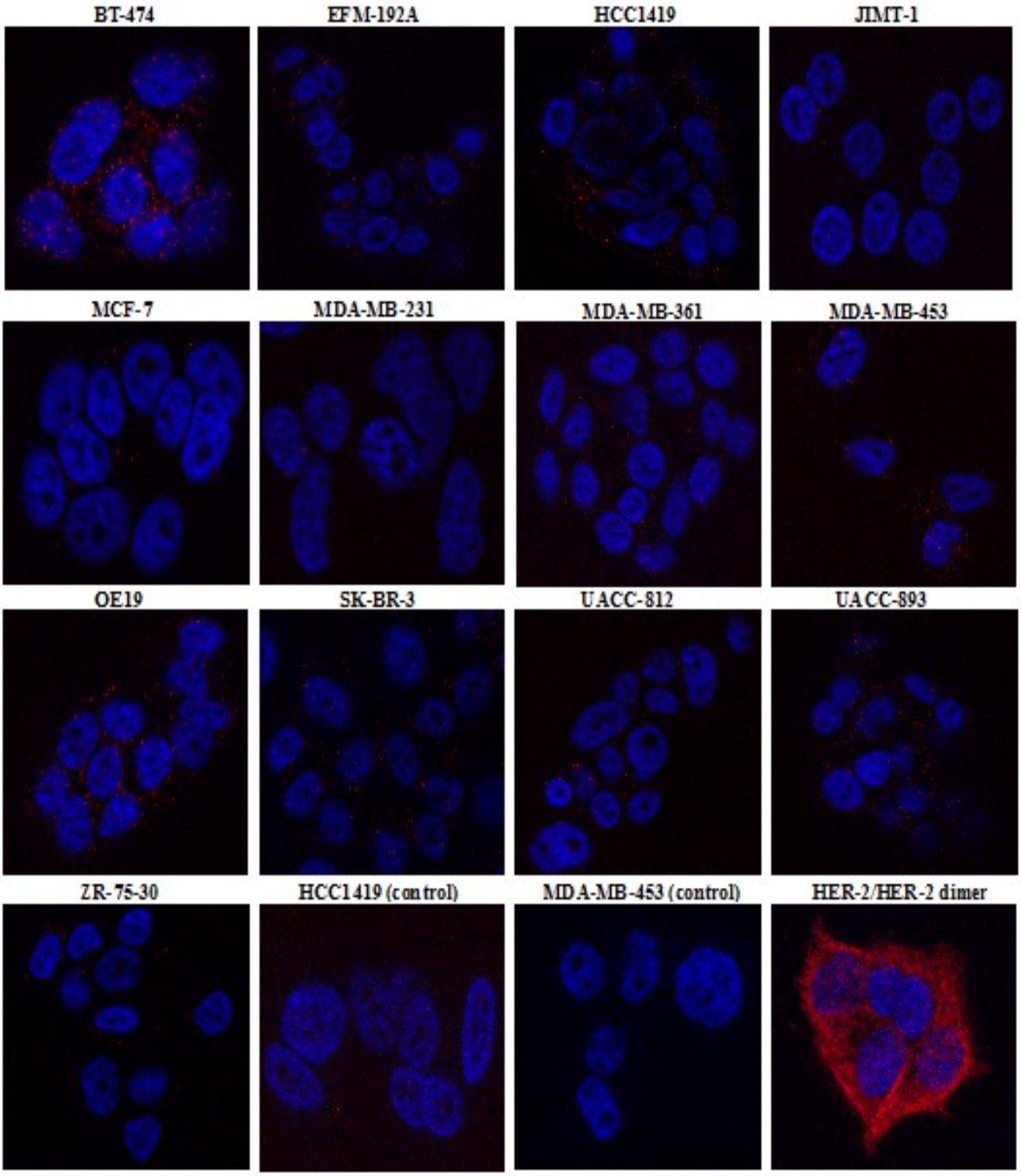

Figure S1. Typical PLA images obtained from each of the studied cell lines. PLA signals are shown in red and the nuclei in blue. Each individual red dot represents one HER-2/HER-3 dimer. Middle images (HCC1419 (control) and MDA-MB-453 (control)) in the bottom row were obtained from negative control samples. In negative samples, only one primary antibody (anti-HER-2) was used. The rightmost image in the bottom row shows an image from a sample in which two diffent anti-HER-2 antibodies, instead of a pair of anti-HER-2 Ab and anti-HER-3 Ab, were used. Two different anti-HER-2 antibodies can bind to the same receptor and, as expected, the detected signal in the HER-2/HER-2 dimer image is considerably stronger than in any other image. This is because two different antibodies which recognize the same protein can generate signals even if no actual dimers are present.

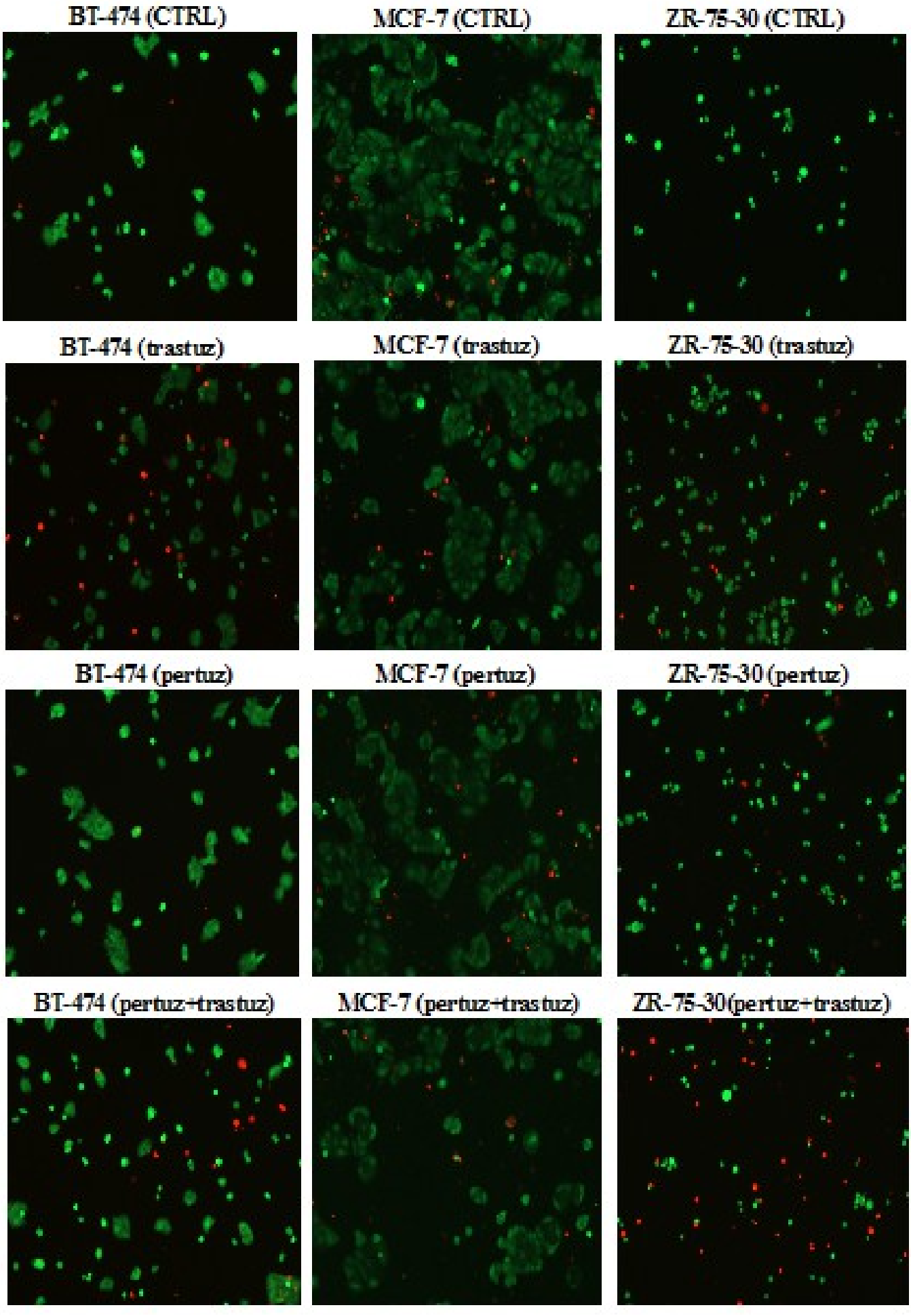
BT-474 (CTRL)
MCF-7 (CTRL)
ZR-75-30 (CTRL)
BT-474 (trastuz)
MCF-7 (trastuz)
ZR-75-30 (trastuz)
BT-474 (pertuz)
MCF-7 (pertuz)
ZR-75-30 (pertuz)
BT-474 (pertuz+trastuz)
MCF-7 (pertuz+trastuz)
ZR-75-30(pertuz+trastuz)

Figure S2. Example images from three different cell lines used in the drug sensitivity assay. Green dye stains live cells while red dye stains dead cells. The top row shows images from control samples, the second row shows images from trastuzumab (20 µg/ml) sensitized cells, the third row shows images from pertuzumab (20 µg/ml) sensizited cells, and the bottom row shows images from cells sensitized with both pertuzumab and trastuzumab (both 20 µg/ml). MCF-7 cells are HER-2 negative and thus they don't significantly react to trastuzumab, pertuzumab, or the combination of these drugs. BT-474 cells, on the other hand, are strongly HER-2 positive, but despite this pertuzumab doesn't seem to have any effect on them. Trastuzumab, however, has notable effect on BT-474 cells. ZR-75-30 cells were among the most drug sensitive of the tested cell lines: the control sample has practically no dead cells, while the sample incubated with both pertuzumab and trastuzumab contains lots of red.