KATRI KÖNINKI

HER-2 Positive Breast Cancer

Molecular and epidemiological studies

ACADEMIC DISSERTATION
To be presented, with the permission of
the Faculty of Medicine of the University of Tampere,
for public discussion in the Auditorium of Finn-Medi 1,
Biokatu 6, Tampere, on December 2nd, 2010, at 12 o'clock.

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ACADEMIC DISSERTATION
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www.uta.fi/taju
http://granum.uta.fi

Cover design by
Mikko Reinikka

Acta Universitatis Tamperensis 1567
ISBN 978-951-44-8274-8 (print)
ISSN-L 1455-1616
ISSN 1455-1616

Acta Electronica Universitatis Tamperensis 1016
ISBN 978-951-44-8275-5 (pdf)
ISSN 1456-954X
http://acta.uta.fi

Tampereen Yliopistopaino Oy – Juvenes Print
Tampere 2010

Jos se ois helppoo
oisin tehnyt sen jo
vaan se on vaikeeta
jos se ois helppoo
oisin tehnyt sen jo
ajat sitten uskotko
- P. Hanhiniemi

Tiivistelmä

Rintasyöpä on naisten yleisin syöpä ja yksi tärkeimmistä kuolinsyistä. Erityyppisten rintasyöpien ennusteet ja hoitovasteet kuitenkin poikkeavat toisistaan. HER-2 –syöpägeenin (human epidermal growth factor receptor) monistuman on todettu olevan yhteydessä aggressiiviseen tautityyppiin. Huolimatta tämän geenimonistuman keskeisestä merkityksestä, HER-2 –positiivisen rintasyövän ilmaantuvuutta ei ole tarkkaan tiedetty. Tällä hetkellä HER-2 -positiiviseen rintasyövän hoitoon on käytössä kaksi täsmälääkettä: humanisoitu monoklonaalinen vasta-aine trastutsumabi sekä HER-1:n ja HER-2:n tyrosiinikinaaseja salpaava lapatinibi. Vaikka näillä lääkkeillä on tärkeä kliininen merkitys, eivät kaikki potilaat kuitenkaan vastaa hoitoon, vaan ovat joko täysin tai osittain resistenttejä lääkkeille. Resistenssimekanismeja on tutkittu, mutta niitä ei vielä toistaiseksi tarkkaan tunneta.

Tässä tutkimuksessa selvitettiin HER-2 -positiivisen rintasyövän osuutta kaikista Pirkanmaan sairaanhoitopiirin alueella vuosina 1982-2005 diagnosoiduista invasiivisista rintasyövistä. Kaikkiaan rintasyövän ilmaantuvuus tänä aikana oli kasvanut 40 %, sen sijaan HER-2 -positiivisen rintasyövän osuus kaikista syövistä oli laskenut noin kolmanneksen. Tämän perusteella voitiin päätellä, että HER-2 -positiivisen rintasyövän ilmaantuvuus oli pysynyt lähes muuttumattomana. Tutkimus osoitti myös, että HER-2 -positiiviset rintasyövät havaittiin sseulontamammografioissa harvemmin kuin HER-2 –negatiiviset kasvaimet.

HER-2 -positiivisen rintasyövän resistenssimekanismeja trastutsumabille tutkittiin JIMT-1 -solulinjalla, joka on eristetty trastutsumabille täysin resistentistä rintasyöpäpotilaasta. Tätä tutkimusmallia verrattiin joukkoon muita HER-2 -positiivisia, trastutsumabille herkkiä rintasyöpäsolulinjoja. Solulinjoista analysoitiin sekä tunnettuja molekulaarisia resistenssimekanismeja, että trastutsumabin aikaansaamaa immunologista ADCC –reaktiota (vasta-aineesta riippuvainen soluvälitteinen sytotoksisuus). Lääkeherkkyyskokeet osoittivat, että trastutsumabi esti jossain määrin lähes kaikkien solulinjojen kasvua. Lapatinibin kyky estää solujen kasvua oli suurempi, tällä lääkkeellä kaikilla solulinjoilla havaittiin IC50 –vaikutus (=lääkekonsentraatio, jossa aiheutuu 50 % solujen kasvun esto). JIMT-1 -solulinja osoittautui resistenteimmäksi molemmille lääkkeille ja se myös ilmensi useita resistenssimekanismeja. Missään muussa solulinjassa ei havaittu kaikkia mekanismeja, vaan niissä eri mekanismeja esiintyi vaihtelevasti. Sen sijaan ADCC –reaktio oli lähes samantasoinen kaikissa tutkituissa HER-2 -positiivisissa solulinjoissa.

Solulinjoilla tehty tutkimus vahvisti käsitystä poikkeavan PI3K-PTEN -signaloinnin merkityksestä HER-2 –positiivisen rintasyövän lääkeresistenssissä ja näitä häiriöitä analysoitiin

tarkemmin kliinisissä kasvainnäytteissä. *PIK3CA* -mutaatioiden osuus oli noin kolmannes kaikissa tutkituissa rintasyövissä. HER-2 -positiivisista rintasyövistä mutaatio havaittiin 14,5 %:ssa ja eloonjäämisanalyysien perusteella tämä melko harvinainen syöpätyyppi osoittautui erittäin aggressiiviseksi. Tarkasteltaessa koko aineistoa havaittiin, että *PIK3CA* -mutaatiot liittyivät myöhäiseen rintasyöpäkuolleisuuteen, tämä ilmiö oli nähtävissä myös estrogeenireseptoripositiivisten kasvainten ryhmässä.

Tutkimus osoitti, että HER-2 –geenimonistuma on rintasyövässä harvinaisempi kuin tähän mennessä on arvioitu. Tulosten perusteella voitiin myös päätellä, että yleisesti tunnetut rintasyövän riskitekijät saattavat altistaa enemmän HER-2 -negatiiviselle syövälle. Analysoitaessa resistenssiä HER-2:n täsmälääkkeitä kohtaan havaittiin, että pääsääntöisesti eri solulinjat ilmensivät resistenssimekanismeja eri tavoin. Lääkeresistenssi on siis monimutkainen, useammasta kuin yhdestä tekijästä johtuva ilmiö. Tutkimus vahvisti PI3K- ja PTEN– signalointihäiriöiden merkitystä sekä trastutsumabi- että lapatinibi-resistenssissä. Lisäksi havaittiin, että mutatoitunut *PIK3CA* saattaa olla biomarkkeri myöhäiselle rintasyöpäkuolleisuudelle, jonka taustalla olevia syitä ei toistaiseksi juurikaan tunneta.

CONTENTS

LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following articles referred to in the text by their Roman numerals:

I Köninki K, Tanner M, Auvinen A, and Isola J (2009): HER-2 positive breast cancer: decreasing proportion but stable incidence in Finnish population from 1982 to 2005. Breast Cancer Res 11:R37.

II Köninki K, Barok M, Tanner M, Staff S, Pitkänen J, Hemmilä P, Ilvesaro J, and Isola J (2009): Multiple molecular mechanisms underlying trastuzumab and lapatinib resistance in JIMT-1 breast cancer cells. Cancer Letters 294:211-219.

III Köninki K, Tanner M, Hemmilä P, Pitkänen J, Tuominen VJ, and Isola J (2010): Distinct pattern of breast cancer specific survival in breast cancers with PIK3CA mutation. Submitted for publication.

ABBREVIATIONS

ADCC	antibody-dependent cellular cytotoxicity
AIB1	amplified in breast cancer 1
AKT	protein kinase B
ALDH	aldehyde dehydrogenase
BAC	bacterial artificial chromosome
BMI	body mass index
BRCA1	breast cancer susceptibility gene1
BRCA2	breast cancer susceptibility gene 2
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CISH	chromogenic in situ hybridization
CD	cluster of differentiation
CDK	cyclin-dependent kinase
DAB	3,3-diaminobenzidine
DCIS	ductal carcinoma in situ
DHPLC	denaturing high performance liquid chromatography
DM1	derivative of maytansine 1
DNA	deoxyribonucleic acid
ECD	extracellular domain
EGF	epidermal growth factor
ER	estrogen receptor
FBS	fetal bovine serum
FGFR	fibroblast growth factor receptor
FITC	fluorescein isothiocyanate
FC	fragment, crystallizable
FDA	food and drug administration
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
FISH	fluorescence in situ hybridization
FOX03	forkhead box O3
HER	human epidermal growth factor receptor
HRT	hormone replacement therapy
IC50	half maximal inhibitory concentration
IG	immunoglobulin
IGF-1R	insulin-like growth factor receptor I
IHC	immunohistochemistry
IRS1	insulin receptor substrate 1
K	keratin
Ki-67	cell cycle related nuclear protein
LDH	lactate dehydrogenase
LOH	loss of heterozygosity
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
MAPK	mitogen activated protein kinase
MUC4	mucin 4, cell surface associated
NRG	neuregulin
NK	natural killer
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline

PDK1	phosphoinositide-dependent kinase 1
PI3K	phosphoinositide 3-kinase
PIK3CA	phosphatidylinositol 3-kinase, catalytic, alpha polypeptide
PIK3R1	phosphoinositide-3-kinase, regulatory subunit 1
PIP	phosphatidylinositol phosphate
PMD	percentage mammographic density
PR	progesterone receptor
PTEN	phosphatase and tensin homolog
qRT-PCR	quantitative real time polymerase chain reaction
RAS	rat sarcoma
RNA	ribonucleic acid
RTK	receptor tyrosine kinase
STK11	serine/threonine kinase 11
SMCC	sulfosuccinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate
TP53	tumor protein p53
UTP	deoxyuridine triphosphate
VEGF	vascular endothelial growth factor
WHO	world health organization

ABSTRACT

Breast cancer is the most common cancer and an important cause of mortality in women. There is, however, vast variation in the aggressiveness of different tumor types. HER-2 (human epidermal growth factor) amplification is shown to correlate with poor prognosis in breast cancer. Despite its prognostic and predictive importance, until now the precise incidence of HER-2 amplification in population-based cohorts of breast cancer has not been known. Currently two targeted drugs (humanized monoclonal antibody trastuzumab and dual kinase inhibitor lapatinib) are accepted for treatment of HER-2 positive breast cancer. Although these two drugs are of significant clinical importance, not all patients benefit from the treatment. The resistance mechanisms have been studied but are until now unclear.

The proportion of HER-2 positive breast cancers was studied during years 1982-2005 in the Pirkanmaa hospital district. The incidence of all breast cancers increased by 40% during the study period when the proportion of HER-2 positive breast cancer of all diagnosed invasive breast cancers declined by third. This indicates that the incidence of tumors with HER-2 amplification had remained almost stable. The results also showed that HER-2 positive breast cancers were underrepresented among the tumors diagnosed by mammography screenings.

Trastuzumab resistance mechanisms were studied in detail using the JIMT-1 breast cancer cell line. This model of intrinsic resistance was compared with a panel of trastuzumab sensitive breast cancer cell lines. All cell lines were characterized for several molecular resistance mechanisms and the potency of the ADCC (antibody-dependent cellular cytotoxicity) reaction evoked by trastuzumab. Trastuzumab induced inhibition of growth in most of the HER-2 positive cell lines studied. Lapatinib produced a more significant action on the HER-2 positive cell lines and the IC50 effect (half maximal inhibitory concentration) was achieved with this drug in all the HER-2 positive cell lines. JIMT-1 being the most resistant to both drugs did also express several co-existing resistance mechanisms while in the more sensitive cell lines these features were present at variable levels. ADCC reaction by normal lymphocytes was nearly equally strong in all HER-2 positive cell lines.

Results of the cell line study further confirmed the role of aberrant PI3K-PTEN signaling in resistance to HER-2 targeted drugs. These defects were studied in more detail in clinical breast tumors. *PIK3CA* mutations were found in third of the analyzed tumor samples. In HER-2 positive cancers the mutation rate was only 14.5% but according to survival analysis this tumor type showed to be aggressive. When considering all breast cancers analyzed, the results

indicated that mutated *PIK3CA* is associated with the trend towards late mortality, also in the estrogen receptor positive tumors.

The study indicated that HER-2 amplification is not as common among breast cancer patients as has been estimated. The results also suggested that the common breast cancer risk factors presumably affect more the HER-2 negative tumor type. The *in vitro* analyses showed that the HER-2 positive cell lines expressed trastuzumab and lapatinib resistance mechanisms at different levels, which proposes that drug resistance is a complicated phenomenon resulting from different biological properties. This study also emphasized the role of aberrant PI3K and PTEN signaling in resistance to both HER-2 targeted agents studied. The results may also implicate that the patients with *PIK3CA* mutated breast cancer are in higher risk of late breast cancer mortality.

INTRODUCTION

According to WHO estimates, cancer is a major health problem and the worldwide implication is still increasing. Cancer incidence is increasing and death rates are expected to grow tenfold during the next decade (Coughlin and Ekwueme, 2009). A similar trend can be seen when considering breast cancer. Despite recent declines in incidence and death rates in many western countries, breast cancer is still an important cause of mortality in women. The survival times vary between patients and depend on tumor burden at the time of diagnosis, tumor characteristics, and efficacy of the treatment. In Finland breast cancer is the second leading cause of death among working-age women (Statistics Finland, 2009). Since it is such an important disease throughout the world, breast cancer risk factors have been under intensive research and the results have shown that breast cancer is a multifactoral disease. The known risk factors are related to exposure of both endogenous and exogenous estrogen as well as reproductive and genetic factors. Several studies have also shown lifestyle and environmental features to be involved with breast cancer risk.

Prognostic and predictive markers provide information for prognosis of the disease, treatment efficacy and resistance. Established markers in breast cancer are tumor size, lymph node status, histological type and histological grade of the tumor. Important molecular factors are estrogen and progesterone receptors and HER-2.

Patients with breast cancer are usually treated with surgery, radiation therapy, chemotherapy and hormonal therapy. Problems of these conventional treatments are frequently occurring side effects as well as development of drug resistance. These disadvantages result from the untargeted cytotoxic effects towards all proliferating cells. High number of evidence from several studies has however shown that breast cancer is a complicated disease at both molecular and clinical level (Perou et al. 2000, Sørlie et al. 2001). Identification of molecular variety of tumors with distinct clinical outcomes and responses to treatment has already led to development of several kind of targeted therapy promising high therapeutic efficacy with minimal side effects. Currently breast cancers are classified in four categories based on expression of hormone receptors and HER-2. These subtypes are luminal A/B (hormone receptor positive, HER-2 negative/HER-2 positive), HER-2 amplified (hormone receptor negative, HER-2 positive) and triple negative (hormone receptor negative, HER-2 negative). To be able to develop more of these effective tailored drugs requires further understanding of molecular pathways aberrant in tumor cells and suitable for drug development. Targeting of the tumor cell specific defects leads to improved therapeutic response, as well as the choice of patients most likely to benefit from the therapy. Although according to the results of several preclinical studies the number of genes mutated in breast cancer is high, mostly

the frequency of mutations in sporadic tumors is low. However, there are exceptions such as components of the PI3K-PTEN pathway, which is an important cell survival pathway also in normal cells.

Until now, three targeted drugs have been accepted for treatment of breast cancer. Two of them are directed against receptor tyrosine kinases of the HER family and one is an inhibitor of vascular endothelial growth factor. Although specific for certain tumor types, the novel, targeted drugs are not always as effective as assumed and therapeutic resistance remains as one of the major unsolved problems in breast cancer. For instance it has been shown in both preclinical and clinical studies that dependence of tumor cells on a certain oncogenic pathway can be bypassed by dysregulation of some other signaling cascade and further leading to reduced drug response. One example is increased activation of the PI3K-PTEN pathway by overexpression of receptor tyrosine kinases, such as HER-2. Increased understanding of resistance mechanisms would result in development of either new therapeutic agents or effective combinations of existing drugs to overcome the resistance. Identification of patients with resistance mechanisms can also lead to discovery of clinically validated biomarkers helping to choose appropriate treatment regimens.

This study aims to describe the epidemiology of HER-2 positive breast cancer. Other main objective is to analyze mechanisms behind resistance to HER-2 targeted therapy, particularly concentrating on the aberrations in PI3K-PTEN pathway. Additionally this research aims to clarify the clinicopathological and prognostic characteristics of breast tumors with defects in this signaling cascade.

REVIEW OF THE LITERATURE

1. Breast cancer incidence

Cancer incidence is the number of new cases occurring in a population during a defined period of time. Although there is a remarkable difference in the incidences of breast cancer between industrialized countries and the developing world, breast cancer is the most common cancer in women and worldwide more than one million women will be diagnosed with breast cancer annually. The breast cancer rates have risen about 30% during the past 25 years in western countries (Garcia et al. 2007). Also in Finland the increase in breast cancer incidence has been remarkable during the recent decades. When the age-adjusted incidence was 28.8/100 000 in the beginning of the 1960s, year 2007 it was 87.5/100 000. Now the trend is expected to decelerate, the predicted increase in the age-adjusted incidence by 2020 is only 3.2% (Finnish Cancer Registry, 2009). The trend is similar in all industrialised countries. The study published 2007 by Ravdin et al. reported a sharp decline in breast cancer incidence in US after year 2003, while before that the incidence had been increasing intensely for twenty years (Glass et al. 2007, Ravdin et al. 2007). In developing world the trend is inverse; breast cancer incidence is increasing. Nevertheless, the incidence rates are still remarkably lower when compared with US and most of the western European countries (Jemal et al. 2010).

1.1 Risk factors for breast cancer

The causative factors for breast cancer are not known, but several factors related to increased risk of breast cancer have been identified. These factors are related to reproductive behavior as well as hormonal and genetic characteristics. Lifestyle and environmental features are also involved. The most important risk factor for breast cancer is female sex, practically all cases occur in women. Also age is a predisposive factor; breast cancer is more common in older women. Breast cancer incidence is highest among 50-59 years old women but declines after that. However, in Finland the incidence is increasing among women aged 60-69 years. Currently the average age at diagnosis is 61 years (Finnish Cancer Registry, 2009).

Family history of breast cancer is a major risk factor. Nevertheless, only 5-10% of all breast cancers can be considered as hereditary. The most important defects causing genetic breast cancer are mutations in *BRCA1* and *BRCA2* genes, which are responsible for 25-30% of hereditary cases (Honrado et al. 2006). Other known genes such as *TP53*, *PTEN* or *STK11* are also associated

with breast cancer susceptibility but explain less than 1% of hereditary breast cancers, respectively. Therefore more than half of familial breast cancer remains unresolved by any of the known genes (De Grève et al. 2008).

Lifetime accumulated hormonal exposure is an essential risk factor of breast cancer. Early menarche, late age at first birth and nulliparity are all related to the high circulating estradiol levels (MacMahon 2006). Postmenopausal obesity increases the amount of estrogen because androgen precursor androstenedione is converted to estrogen in adipocytes (Stoll 2000). Exogenous exposure to estrogen is also shown to increase risk of breast cancer. Use of oral contraceptives has been linked to a slightly increased risk or even no risk for breast cancer (Marchbanks et al. 2002). The association between post-menopausal hormone replacement therapy (HRT) and breast cancer was first published in a pooled analysis (Collaborative Group on Hormonal Factors in Breast Cancer 1997) and has later been confirmed in several studies (Collins et al. 2005, Lee et al. 2005b). However, the relation between HRT and breast cancer is complicated. The risk depends on the regimens used and length of HRT therapy (Collins et al. 2005) although recent results show that even shorter duration of therapy may increase the risk of breast cancer (Fournier et al. 2009). Due to different practices of HRT use in different countries its association with breast cancer may also differ internationally. It has been suggested that there are differences in the hormone-related risk factors between the different breast cancer subtypes (Ma et al. 2010). Tumors of HRT users are also known to be more often smaller, local and of lower histological grade (Holli et al. 1998, Cheek et al. 2002, Schuetz et al. 2007).

Breast density expressed as the percentage mammographic density (PMD) is shown to be an independent breast cancer risk factor (Boyd et al. 2009). Dietary factors including alcohol intake and lack of physical activity have also been studied as possibly predisposing to breast cancer (Key et al. 2003, Friedenreich and Cust 2008). There are some recent publications proposing a role for use of bisphosphonates in the reduced risk of breast cancer (Chlebowski et al. 2010, Newcomb et al. 2010, Rennert et al. 2010). One possible explanation for this observation is the association between low bone density and lower risk of breast cancer because bisphosphonates are commonly used for the treatment of osteoporosis and for prevention and treatment of skeletal lesions due to malignancy. However, the exact role of these agents in breast cancer prevention is not yet clear.

Alterations in the exposure of women to the risk factors reflect also to the incidence of breast cancer. Changes in the HRT use have been reported in several countries. In the 1980s the use of HRT increased and estrogen was changed to estrogen-progestin products (Stefanick 2005). Results of The Million Women Study (Beral et al. 2003) and The Womens' Health Initiative (Rossouw et al. 2002) showed a significant association between breast cancer risk and use of estrogen–progestin combination therapy. Consequently in US the use of HRT decreased by 38% by

the end of 2002 (Ravdin et al. 2007). In Finland the decline was 25% between years 2001 and 2005 (Hemminki et al. 2008). These changes in therapy practices have also been reported to reflect the breast cancer incidence in several countries (Katalinic and Rawal 2008, Vankrunkelsven et al. 2009, Lambe et al. 2010).

In addition to risk factors, also diagnostic methods used influence breast cancer incidence. Screening mammography was implemented in western countries in mid-1980s and at present breast cancer screenings are performed widely (Schopper and de Wolf 2009). The national mammography screening programme was introduced in Finland in 1987. From 1992 onwards all women aged 50-59 years were invited to screening every two years. In 2007 the invitational age was extended to reach all 50-69 years by the year 2016 (Finnish Cancer Registry, 2009). The coverage of the screenings in Finland is 95-100% (Sarkeala et al. 2008). Adoption of screening program leads to major increase in the incidence of breast cancer due to detection of slow-growing tumors that have accumulated over several years. Although the incidence rates decline in the end, the incidence remains at higher level compared with unscreened populations (Jonsson et al. 2005, Zackrisson et al. 2006). This is caused by detection of small, slow-growing tumors by screenings that might probably never have been diagnosed clinically. Also the lead-time bias, which is the length of time between the detection of a tumor by screening mammography and its usual clinical presentation, affects the incidence rates by lowering the age at diagnosis (Biesheuvel et al. 2007).

1.2 Recent changes in breast cancer incidence

Possible alterations in risk factors and improved diagnostic methods may not have an equal effect on all breast cancer subtypes. At the time of diagnosis breast cancers are characterized according to tumor size, axillary lymph node and distant metastasis status and the histologic grade. The increase in the incidence of breast cancer beginning from the 1980s can be explained mostly by localized breast cancers, while the incidence of advanced cancers has not had a similar rate of increase (Hofvind et al. 2008). Incidence of in situ carcinomas has increased more than invasive breast cancers, partly due to increased diagnosis by mammography screenings (Hofvind et al. 2008, Virnig et al. 2009).

Also hormone receptors and HER-2 status are determined for all primary breast cancers. More than 75 % of invasive breast cancers are estrogen receptor positive and a slightly smaller proportion are also progesterone receptor positive (Ferté et al. 2010). There are few studies evaluating breast cancer trends by hormone receptor status (Glass and Hoover 1990, Pujol et al. 1994, Li et al. 2003) and showing a significant increase in the proportion of estrogen receptor-

positive tumors. However, the estrogen receptor assay method has changed during the last 30 years and at the same time the sensitivity of the assay methods used has improved. The possible bias caused by this is impossible to determine retrospectively. While the incidence of lobular breast cancer and tumors of smaller size has increased by the screenings, the trend for ER negative tumors has been downward from the 1980s (Hofvind et al. 2008). However, the lately observed declining trend concerns various types of tumors. In US the recent decrease in breast cancer incidence is related to women aged 50 years or more and specifically to ER positive cancer (Glass et al. 2007, Ravdin et al. 2007). The downward trend is seen also with localized and regional cancers while the proportion of distant tumors has increased (Glass et al. 2007). Until now the time trends in the incidence of HER-2-positive breast cancer have remained unclear.

1.3 Breast cancer survival and mortality

The survival time of a patient is the time between diagnosis and death. Despite the high incidence rates, the prognosis of breast cancer is good when compared with many other cancers and the survival rate is high, the five-year survival being 89% in Finland during years 2003-2005 (Finnish Cancer Registry, 2009). However, unlike with many other cancers, in breast cancer the survival curve does not flatten out within 20 years after diagnosis. Proportion of patients alive at 15 years after diagnosis is under 50% (Brenner and Hakulinen 2002). Although still being the leading cause of cancer mortality in women, breast cancer is only the fifth cause of death from cancer overall, (Parkin and Fernández, 2006). In Finland the age-adjusted breast cancer mortality has remained equal from 1962 (14.0/100 000 person-years) until 2008 (13.5/100 000 person-years) although being at a higher level during 1980-2000. Mortality rates are predicted to decrease by 24 % by year 2020 (Finnish Cancer Registry, 2009). In addition to patient survival, mortality predictions are affected by incidence rates.

Randomized prospective trials have shown that mammography screenings decrease breast cancer mortality with a 10 years delay and this has been observed also in Finland. However, in the long run early detection of tumors decreases mortality only if treatment of these tumors is more effective than tumors diagnosed at later stage. Reduction in mortality is also caused by earlier detection of palpable tumors and introduction of novel chemotherapeutic agents as well as targeted therapies (Jatoi and Miller 2003). The first chemotherapeutic agents were invented already in the mid 1900s and the first clinical studies about adjuvant chemotherapy were published in 1970s (DeVita and Chu 2008). Adjuvant therapy is chemotherapy given after surgery of primary tumor aiming to target tumor cells that could form metastases in the future. In the 1980s results from

several large randomized trials were published indicating a 20% decrease in breast cancer mortality by the use of adjuvant systemic therapy, which is use of chemotherapy, hormone therapy and/or targeted therapy or a combination of these. By the end of the decade tamoxifen, an antagonist of the estrogen receptor in breast tissue used for the treatment of estrogen receptor positive tumors, and polychemotherapy were widely in use as adjuvant therapy. Adjuvant therapy is considered a major factor in lowering breast-cancer mortality (Jatoi and Miller 2003). Development of new chemotherapeutic agents is still ongoing. The non-specific action and side effects of these drugs as well as active research has led to development of targeted therapy. The anti-HER-2 monoclonal antibody trastuzumab for the treatment of breast cancer was approved 1998 and currently used in clinical practice are also the dual HER-1/HER-2 tyrosine kinase inhibitor lapatinib, and the anti-vascular endothelial growth factor (VEGF) antibody bevacizumab. Whether these targeted therapies are able to reduce breast cancer mortality will become evident in the future.

Although the trend in breast cancer incidence is stabilizing, the prevalence of breast cancer, which is the total number of cases alive, remains high due to favourable survival rates. In 2008 over 50 000 Finnish women were alive with breast cancer. The prevalence is predicted to remain at the same level also for the next decade (Finnish Cancer Registry, 2009). Therefore breast cancer is an important target of treatment and research. Increasing knowledge of biology of breast cancer enables the development of more specific drugs.

2. Origin of breast cancer

Human breast cancers are heterogeneous in their morphology, response to therapy and clinical course. Therefore the cascade of genetic alterations in the development of breast cancer is complex and not well known until now. Previously breast cancer progression was seen as a multi-step process, according to the Vogelstein's model for colon carcinogenesis (Vogelstein et al. 1988), involving progressive changes from normal to hyperplasia with and without atypia, carcinoma *in situ*, invasive carcinoma, and metastasis (Shackney et al. 2003). This is probably at least partially a too simplistic view. Recent immunohistochemical and molecular genetics studies have shown that development of breast cancer does not follow a single pathway but is a complex series of random genetic events leading towards invasive breast cancer. It has been shown that low and high grade breast cancers differ in the quantity but also type of genomic alterations suggesting that tumors do not always progress from low to high grade. Studies have also shown that the variation in genetic aberrations is wider between tumors of different grade than of different histological type (Reis-Filho and Lakhani 2003).

The cancer stem cell model postulates that cancer originates from stem cells, as a consequence of dysregulation of self-renewal pathways. This implicates that tumors contain a subpopulation of cells with stem cell like properties, which are capable of self renewal and differentiation. Presence of stem cells promotes tumorigenesis and cellular heterogeneity and this cell population is expanding (Charafe-Jauffret et al. 2008). Putative cancer stem cells have been isolated also from breast cancers (Al-Hajj et al. 2003) and these cells are shown to be resistant to chemotherapeutic drugs (Dave and Chang 2009).

2.1 Tumor suppressor genes

Inactivation of tumor suppressor genes is an important event in the origin of many cancers. Tumor suppressor genes are involved in the cell cycle, DNA repair, the metabolism of carcinogens, cell-to-cell interaction, apoptosis and angiogenesis. Tumor suppressor genes are categorized into two groups, gatekeepers and caretakers (Kinzler and Vogelstein 1997). The gatekeepers are directly involved in controlling proliferation by regulating cell cycle checkpoints. The PTEN (phosphatise and tensin homolog) tumor suppressor gene analyzed in this study is one example of the gatekeepers. The caretakers instead have an indirect effect on growth. They are responsible for genome integrity and changes in these genes lead to genome instability. Tumor suppressor genes are considered to act mostly in a recessive fashion, meaning that abnormality must affect both gene alleles. The classical inactivation of tumor suppressor genes is the so-called Knudson 'Two-Hit' hypothesis (Knudson 1971). It is caused by tumor suppressor gene loss due to chromosomal deletion of one allele and mutation of the other remaining allele. Tumor suppressor genes can be inactivated also by epigenetic changes, which alter gene expression and chromatin organization without a change in the DNA sequence. Most important of these changes are DNA methylation, histone methylation and deacetylation (Probst et al. 2009).

2.2 Oncogenes

Oncogenes encode proteins that control cell proliferation, apoptosis, or both. They can be activated by structural alterations resulting from mutation or gene fusion (Konopka et al. 1985), by juxtaposition to enhancers (Tsujimoto et al. 1985) or by amplification. Translocations and mutations can occur either as initiating events or during tumor progression while amplification usually occurs during tumor progression. Mutations change the structure of protein encoded in a way that enhances its transforming activity. Protein products of oncogenes can be derived into different groups;

transcription factors, chromatin remodelers, growth factors, growth factor receptors, signal transducers and apoptosis regulators. Oncogenes are also important as therapeutical targets (Croce 2008).

2.3 Cell signaling in cancer

Protein products of tumor suppressor genes and oncogenes constitute several signaling pathways in cells. These pathways are essential for the normal functions of cells. Different signaling pathways form complex networks and the cross-talk between these cascades enable extracellular signals to evoke several cell biological effects. In cancer the deregulation of these pathways is of crucial importance. There are certain major pathways affected either directly or indirectly by oncogenic alterations. These are the pathways regulating cell proliferation, migration, apoptosis and angiogenesis (Christoffersen et al. 2009).

3. HER-2 oncogene

One of the most important targets of therapy in breast cancer is HER-2 encoded by the *HER-2* gene (also called *c-erbB-2* or *neu* according to the corresponding rat oncogene), located in chromosome 17 (Schechter et al. 1984, King et al. 1985). HER-2 belongs to the family of transmembrane receptor tyrosine kinases with HER-1, HER-3 and HER-4. These receptors are expressed in various tissues of epithelial, mesenchymal and neuronal origin. Functional HER-2 is shown to be important both in formation of normal cardiac tissue during embryogenesis (Lee et al. 1995) and maintenance of adult heart (Crone et al. 2002) as well as differentiation of oligodendrocytes in the spinal cord (Park et al. 2001). The physiological role of HER receptors in breast is to contribute to postnatal development. It has been shown that knock-out mice without HER-2 expression lack formation of lobular structures, lobular expansion and production of milk (Hynes and Lane 2005). HER-2 is also an important regulator of cell growth and differentiation during puberty (Olayioye et al. 2000).

All members of this family have common physiological characteristics, such as an extracellular domain, which in HER-1, HER-3 and HER-4 binds ligands, a transmembrane domain and an intracellular protein tyrosine kinase domain. However, the three ligand-binding receptors have different ligands, differences in their enzymatic activities and different affinities for downstream signaling molecules (Olayioye et al. 2000, Yarden and Sliwkowski 2001). Although the receptor tyrosine kinases are membrane proteins, HER-1, HER-2, HER-3 and the C-terminal fragment of HER-4 have been reported to be localized also in the nucleus of cancer cells. Functions

of the nuclear proteins are shown to involve transcriptional regulation in several ways (Wang et al. 2010).

The HER family members are activated by several different peptide ligands. The activating ligands of HER-1 include EGF (epidermal growth factor), TGF-α (transforming growth factor-α), AR (amphiregulin), HB-EGF (heparin-binding EGF like growth factor), BTC (betacellulin), EPR (epiregulin) and EPG (epigen) (Riese et al. 1996, Strachan et al. 2001). HER-4 binds the family of neuregulins (NRG-1, NRG-2, NRG-3, NRG-4) and additionally BTC, EPR and HB-EGF (Carraway et al. 1997, Elenius et al. 1997, Harari et al. 1999). HER-3 has only two ligands, NRG-1 and NRG-2 (Chang et al. 1997). The ligands exist as membrane-bound and are released in soluble form through ectodomain shedding, meaning that the extracellular domain of ligands is cleaved by proteases. The ligands can act in an autocrine (activating receptors on the same cell), paracrine (activating adjacent cells) or endocrine (activating cells in other organs) manner (Lee et al. 2003). Ligand binding leads to conformational change and further to homo- or heterodimerization of the receptors. HER-2 does not bind any ligand but it is activated by heterodimerization with the other receptors (Garrett et al. 2003). Additionally, HER-2 is the preferred dimerization partner and heterodimers including HER-2 are more potent signaling complexes compared with homodimers (Pinkas-Kramarski et al. 1996, Graus-Porta et al. 1997). In contrast to other members of the receptor family, HER-2 is constitutively in the active conformation (Garrett et al. 2003).

Receptor dimerization induces phosphorylation of highly conserved tyrosine residues in the cytoplasmic kinase domains of the proteins (Schlessinger 2000). Phosporylation activates specific molecules whose binding initiates either directly or via crosstalks various downstream signaling pathways crucial for cell survival, cytoskeletal organization, cell cycle progression, and gene transcription (Kruser and Wheeler 2010). The signaling pathways activated depend on the ligand and dimerization partner in question (Kruser and Wheeler 2010) and the different cascades are presented in Figure 1. HER-3 has been shown to be the most important activator of PI3K signaling (Wallasch et al. 1995) and HER-2/HER-3 heterodimers are the most potent prosurvival receptor signaling complexes (Holbro et al. 2003, Lee-Hoeflich et al. 2008). After dimerization and signal transduction HER-2 and the other receptors in the family are internalized and inside the cell they are marked by enzymes for ubiquitination to be destroyed in lysosomes. Occasionally receptors may also be recycled when they return to the cell surface. This increases the potency of the receptors (Baulida et al. 1996, Worthylake et al. 1999).

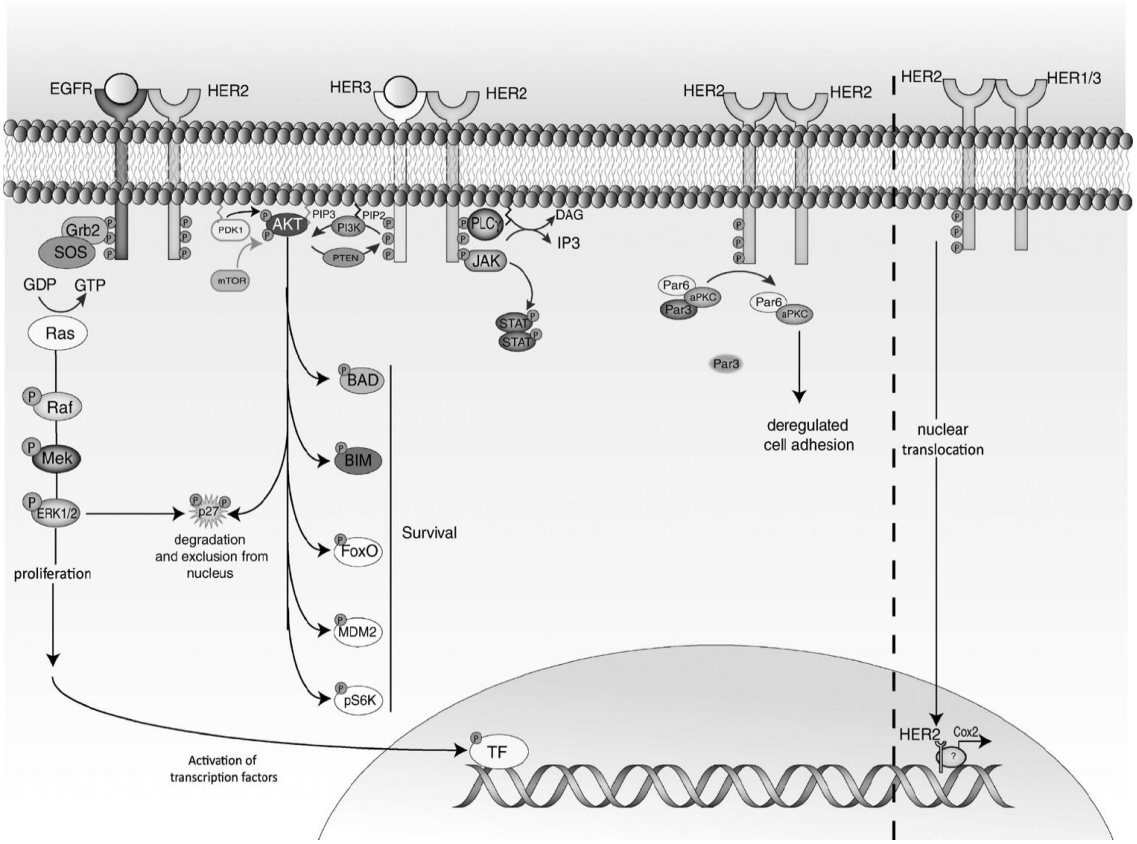

Figure 1. *HER-2 dimerization results in increased downstream signaling through different pathways. HER-1/HER-2 dimers increase signalling in the Ras/Raf/MAKP pathway resulting in activation of transcription factors and further cell proliferation. HER-2/HER-3 heterodimers enable activation of PI3K and AKT. AKT enhances cell survival by several regulator proteins. HER-2 homodimers form a complex with Par and PKC proteins, which in cancer cells decrease cell adhesion and inhibits apoptosis. Nuclear HER proteins associate with DNA-binding transcription factors and thus enhance target gene transcription (Kruser and Wheeler 2010).*

3.1 HER-2 oncogene in breast cancer

HER-2 gene is amplified in 10-20% of breast cancers. Amplification leads to protein overexpression (Slamon et al. 1987, Slamon et al. 1989) and further to aberrant signaling and excessive activation of the signaling pathways. The role of amplified HER-2 in the biology of breast cancer has been shown *in vitro* in several studies. HER-2 overexpression in HER-2 negative MCF-7 breast tumor cells did change biological behaviour of the cells and resulted in increased proliferation, more aggressive invasion and an over twofold increase in the metastatic potential (Ross et al. 2003). Cell line studies have also shown that the level of expression is critical for transformation. NIH3T3 fibroblasts expressing high level of HER-2 are shown to be able to induce tumor formation in athymic mice (Hudziak et al. 1987). There is evidence suggesting that these features partly result from ability of HER-2 to maintain and increase cancer stem cell properties (Korkaya et al. 2008, Magnifico et al. 2009). Increased expression of the ALDH (aldehyde

dehydrogenase) enzyme is considered as a stem cell marker. A positive correlation between ALDH and HER-2 overexpression has been observed also in clinical breast tumors (Ginestier et al. 2007). HER-2 is also proposed to regulate self-renewal and invasion of both normal and malignant human mammary stem cells via PI3K-PTEN pathway (Korkaya et al. 2008).

In clinical breast cancers HER-2 overexpression is more frequently detected in DCIS (ductal carcinoma in situ) than in invasive tumors (Hoque et al. 2002). In invasive breast cancer HER-2 amplification occurs at significantly higher level in ductal carcinomas than in lobular carcinomas (Hoff et al. 2002, Ariga et al. 2005). Several studies have compared the HER-2 status of paired primary and metastatic tumor tissues and most of the studies have shown a consistency (Tanner et al. 2001, Xu et al. 2002, Bozzetti et al. 2003, Tapia et al. 2007). Contradictory results have also been presented (Santinelli et al. 2008), particularly as a result of trastuzumab therapy (Pectasides et al. 2006). HER-2 status is shown to remain unchanged in the primary tumors of most patients after administration of neoadjuvant chemotherapy (Arens et al. 2005, D'Alfonso et al. 2010) while trastuzumab-based neoadjuvant therapy is shown to result in loss of HER-2 amplification (Hurley et al. 2006, Mittendorf et al. 2009). Reasons for this change are not known. It is proposed to be due to heterogeneity of the HER-2 expression within the tumor, suggesting that trastuzumab eliminates HER-2 overexpressing clones leaving only the HER-2 negative tumor cells. Thus it would reflect response to treatment, as well as be a mechanism of resistance since the HER-2 negative tumors no longer respond to trastuzumab (Mittendorf et al. 2009).

3.2 HER-2 as prognostic factor in breast cancer

Prognostic factor is by definition "A situation or condition, or a characteristic of a patient, that can be used to estimate the chance of recovery from a disease or the chance of the disease recurring". HER-2 amplification/overexpression is shown to be an independent prognostic factor in breast cancer associated with poor prognosis. Patients with HER-2 positive cancer are reported to have a shorter disease-free survival and overall survival than patients with normal HER-2 status (Slamon et al. 1987, Slamon et al. 1989, Paterson et al. 1991, Press et al. 1993). HER-2 positive status predicts poor prognosis also in axillary lymph node positive cancer (Slamon et al. 1987, Borg et al. 1990, Rilke et al. 1991, Gusterson et al. 1992, Toikkanen et al. 1992). It has been shown that a large part of patients has tumor cells in bone marrow at the time of diagnosis (Harbeck et al. 1994, Braun et al. 2000, Gebauer et al. 2001). Breast cancer patients with HER-2 positive tumor cells in the bone marrow have a greater risk for metastatic relapse than patients with tumor cells lacking detectable expression of HER-2 (Braun et al. 2001).

Additionally, HER-2 positive breast cancer has been associated with adverse prognostic factors, such as tumor size (Borg et al. 1990, Rilke et al. 1991), axillary lymph node involvement (Borg et al. 1990, Gusterson et al. 1992), tumor stage (Borg et al. 1990, Paik et al. 1990, Rilke et al. 1991, Gusterson et al. 1992), negative hormone receptor status (Borg et al. 1990, Gusterson et al. 1992, Benz et al. 1993, Pietras et al. 1995) increased growth fraction, and high nuclear grade (Andrulis et al. 1998).

Although generally associated with poor prognosis, the outcome of patients with HER-2 positive breast cancer varies, proposing that HER-2 amplified tumors are heterogenous by their biology. A recent gene expression analysis has revealed three subgroups of HER-2 positive cancers with distinct grade, histological stage and ER status as well as differences in survival times. According to this report, only one of these subtypes, mostly consisting of tumors with the ER negative type, was associated with significantly worse outcome. This study identified also a cluster of tumors with basal-like phenotype (Staaf et al. 2010), which, according to some studies is inversely associated with HER-2 amplification (Perou et al. 2000, Sørlie et al. 2003).

3.3 HER-2 as predictive factor in breast cancer

Predictive factor is "a condition or finding that can be used to help predict whether a person's cancer will respond to a specific treatment". HER-2 status predicts response to HER-2 targeted drugs but it has been associated also with other cancer therapies. The correlation between HER-2 status and response of tumors to chemotherapy is still controversial. Some studies show that patients with HER-2 positive breast cancer respond better to anthracycline based treatment when compared with therapies without anthracycline (Paik et al. 1998, Pritchard et al. 2006) while also opposite results have been presented (Pegram et al. 1997, Konecny et al. 2001). The gene encoding DNA modifying enzyme topoisomerase II α is located in close proximity to HER-2 on chromosome 17 and it is shown to be often deleted or amplified in HER-2 positive breast cancer (Järvinen et al. 2000). Increase in topoisomerase II α expression is shown to predict response to adjuvant chemotherapy (Kim et al. 1991, Smith et al. 1993) and therefore it has been proposed that defects in this gene would be the reason for altered anthracyclin response instead of HER-2 amplification. Also activation of the HER receptors is associated with upregulation of topoisomerase II α and increased sensitivity to anthracyclines (Harris et al. 2001). HER-2 has also been shown to be an independent predictor of response to anthracyclin based therapy (Harris et al. 2009).

Increased HER-2 activity has been associated with resistance to hormonal therapy in preclinical (Nicholson et al. 2000, Schafer et al. 2002, Osborne et al. 2003, Shou et al. 2004) and

clinical studies (Borg et al. 1994, Houston et al. 1999). It has been shown that in HER-2 overexpressing cells tamoxifen increases expression of several estrogen-dependent genes, including insulin receptor substrate 1 (IRS1) and cyclin D1, both important for tumor growth. This effect is dependent on cross-talk with the HER-2 pathway (Shou et al. 2004) as a result of phosphorylation of estrogen receptor and its accessory protein amplified in breast cancer 1 (AIB1) via HER-2 activation (Smith 1998). Also AIB1 is often overexpressed in HER-2 positive tumors (Bouras et al. 2001). The outcome is bidirectional cross-talk leading to cell survival and proliferation (Shou et al. 2004). Clinically the resistance is at least partially due to lower level of hormone receptor expression in HER-2 positive tumors (Konecny et al. 2003).

3.4 Methods of assessment of HER-2 oncogene status

Assesment of HER-2 status is part of routine diagnostics in breast cancer and accurate determination is important for identifying the patients who most probably will benefit from the treatment. Several methods are available either for determination of HER-2 protein overexpression or gene amplification. HER-2 protein expression can be analyzed by immunohistochemistry (IHC) when tumor is stained using an antibody directed against HER-2 protein. The results are interpreted using a conventional light microscope. This method is relatively quick and inexpensive. The problems of IHC are intraobserver variability and divergence in the fixation of the samples, which affects staining result. Commonly the weakly positive (a score of +2) results are confirmed by determining the gene amplification (Penault –Llorca et al. 2009).

Fluorescence in situ hybridization (FISH) detects the number of copies of the *HER-2* gene. This method is considered to be more objective and quantitative than IHC. However, FISH is also more expensive and a fluorescence microscope is needed for interpretation of the results. Unlike with immunohistochemistry, the fluorescence signals decay from the slides. Chromogenic in situ hybridization (CISH) is another method for assessment of the *HER-2* gene amplification. Advantages of CISH compared with FISH are the use of standard light microscope for sample analysis and lower cost. The staining also remains for as long as with IHC (Penault –Llorca et al. 2009).

4. HER-2 targeted therapy

Due to its prognostic importance, HER-2 has been a target for intensive drug development. There are several agents in clinical use and under preclinical and clinical trials. These regimens are mostly

antibodies targeted against the extracellular part of the HER-2 protein. Also other kinds of molecules, for example small molecule tyrosine kinase inhibitors, have proven to be effective in the treatment of HER-2 positive breast cancer. Novel treatment strategies include also co-targeting other members of the HER receptor family. The HER-2 targeted drugs already accepted for treatment of HER-2 positive breast cancer or under clinical trials are presented in Table 1.

Table 1.

Drugs in clinical use		
Agent		**Target**
Trastuzumab	monoclonal antibody	HER-2
Lapatinib	tyrosine kinase inhibitor	HER-1/HER-2
Drugs under clinical investigation		
Agent		**Target**
Trastuzumab-DM1	antibody-drug conjugate	HER-2
Pertuzumab	monoclonal antibody	HER-2 (prevents HER-2/HER-3 dimerization)
Ertumaxomab	monoclonal antibody	HER-2/CD3
BIBW 2992	tyrosine kinase inhibitor	HER-1/HER-2
Neratinib (HKI-272)	tyrosine kinase inhibitor	pan-HER
HER-2 vaccines	Different antigens • HER-2 positive cells • HER-2 specific molecules	immune response against HER-2

4.1 Trastuzumab

Trastuzumab is a recombinant humanized monoclonal antibody targeted against HER-2 receptor derived from a murine monoclonal antibody 4D5, which was *in vitro* shown to specifically inhibit proliferation of human breast cancer cells overexpressing HER-2 (Hudziak et al. 1989). Problem with clinical use of rodent monoclonal antibodies is the development of anti-globulin response during therapy. Therefore the antibody was humanized by fusing the antigen binding region from the rodent antibody into the framework region of human immunoglobulins. The humanized antibody was shown to bind HER-2 receptors more tightly and inhibit cell proliferation almost as effectively as the parent antibody and also support cytotoxicity via antibody dependent cell cytotoxicity (ADCC) in HER-2 overexpressing breast cancer cells (Carter et al. 1992). Trastuzumab was shown to have antitumor activity against human breast cancer xenografts both as a single agent and combined with chemotherapeutics (Baselga et al. 1998, Pietras et al. 1998).

In a phase II clinical study the overall response rate of trastuzumab as a single agent was 11.6% in patients with metastatic disease (Baselga et al. 1996). When given to patients who

had progressed after chemotherapy the response rate was nearly the same, 15% in all patients treated, but observed to be better among patients with higher level of HER-2 overexpression (Cobleigh et al. 1999). In a phase II study the use of trastuzumab in combination with the chemotherapeutic chisplatin resulted in higher clinical response rates in pretreated patients (Pegram et al. 1998). Trastuzumab and chemotherapeutic agents together in patients who had not received chemotherapy for metastatic breast cancer were tested in a phase III trial. In this study the rate of overall response for patients treated with combination therapy was significantly higher than previously observed (Slamon et al. 2001). The combination studies also revealed the incidence of cardiotoxicity, which is the most severe side effect of trastuzumab. This had not been observed in previous trials with trastuzumab as a single agent or as combination therapy in animals (Cobleigh et al. 1999, Slamon et al. 2001). Trastuzumab (Herceptin®) was accepted for treatment of HER-2 positive breast cancer year 1998 by FDA in United States and year 2000 by EU in Europe.

After promising results in patients with metastatic breast cancer, trastuzumab was studied also in adjuvant setting. Combining trastuzumab with adjuvant chemotherapy was shown to increase disease free survival significantly (Romond et al. 2005, Piccart-Gebhart et al. 2005). In the FinHer trial combination of trastuzumab and adjuvant chemotherapy was administered for nine weeks instead of the established one year. Also in this trial was seen a significant improvement in disease free survival (Joensuu et al. 2006). At present, trastuzumab is used for treatment of metastatic and early-stage breast cancer along with chemotherapeutic agents (Cobleigh et al. 1999, Esteva et al. 2002, Seidman et al. 2001, Slamon et. al 2001).

4.2 Lapatinib

Lapatinib is an oral small molecule reversible dual inhibitor of HER-1 and HER-2 receptors. It is currently used in combination with the chemotherapeutic capecitabine for the treatment of patients with HER-2 positive metastatic breast cancer who have progressed on trastuzumab-based therapy (Xia et al. 2006, Esteva et al. 2010). In clinical studies lapatinib is shown to be effective also as monotherapy (Cameron et al. 2008, Gomez et al. 2008). Effect of lapatinib combined with trastuzumab or other chemotherapeutic agents than capecitabine in the treatment of metastatic breast cancer has also been studied in clinical setting. The phase III trials have shown that progression-free survival and clinical benefit rates were significantly improved by the combination of lapatinib with trastuzumab when compared with lapatinib alone (O'Shaughnessy et al. 2008). Clinical studies evaluating the use of lapatinib in the adjuvant and neoadjuvant setting are currently ongoing (Nielsen et al. 2009).

Due to smaller molecular size compared with trastuzumab, lapatinib is able to cross the blood-brain barrier and preclinical data suggests that lapatinib may prevent the growth of brain metastases (Gril et al. 2008). Central nervous system metastasis are common in breast cancer and patients with HER-2 positive disease are shown to have these metastasis twice as often than breast cancer patients on average (Altaha et al. 2005, Souglakos et al. 2006). The efficacy of lapatinib for breast cancer with brain metastases has been studied also in clinical setting but until now no significant results have been obtained (Lin et al. 2008, Lin et al. 2009). Lapatinib is shown also to decrease amount of tumor stem cells in neoadjuvant setting (Li et al. 2008).

4.3 Other HER-2 targeting agents

One mechanism for strengthening the effect of trastuzumab is development of antibody-drug conjugates, which are monoclonal antibodies linked to a small cytotoxic molecule. These molecules are supposed to combine the effect of cytotoxic drugs and specificity of antibodies. Trastuzumab-DM1 is a conjugate of trastuzumab and DM1 (derivative of maytansine 1). Trastuzumab is in preclinical studies shown to specifically bind HER-2 receptors, which is important for recognition of target cell. HER-2 receptors are expressed at high levels on tumor cells compared with normal tissue and are known to be internalized by endocytosis, enabling access of the DM1 molecule into cells. DM1 inhibits microtubulus polymerization in cells and thus cell proliferation (Cassady et al. 2004). The linker has an important role in tolerability of the conjugate. Trastuzumab is linked with DM1 via a thioether linker SMCC, which stabilizes the bond and helps to maintain efficacy and decrease toxicity. Trastuzumab-DM1 is shown to be endocytosed by the cancer cell followed by degradation of the conjugate in lysosomes (Lewis Phillips et al. 2008).

Trastuzumab-DM1 showed antitumor activity in HER-2 positive tumor models, even those being resistant to trastuzumab. The phase I clinical trial with patients with metastatic HER-2 positive breast cancer who had previously received a trastuzumab-containing chemotherapy regimen showed a response rate of 44%. The most common side effect observed was thrombocytopenia, which is common in cytotoxic cancer therapy. Cardiotoxicity did not occur. In all, phase I results showed trastuzumab-DM1 to be well-tolerated. The clinical trials have proceeded to phases II and III (Krop et al. 2010).

Pertuzumab is a HER-2 binding monoclonal antibody with a different mechanism of action than trastuzumab. Pertuzumab binds a different epitope of the HER-2 receptor (Whenham et al. 2008) and thereby prevents particularly neuregulin 1 induced HER-2/HER-3 dimerization and further signal transduction (Agus et al. 2002, Franklin et al. 2004, Adams et al. 2006). Combination

of pertuzumab and trastuzumab has been shown to induce apoptosis both *in vitro* and *in vivo* (Nahta et al. 2004a, Lee-Hoeflich et al. 2008, Scheuer et al. 2009). In phase II trials pertuzumab showed activity in patients with trastuzumab resistant metastatic breast cancer (Esteva et al. 2010). Pertuzumab is currently under phase III clinical studies in combination with trastuzumab and docetaxel in previously untreated patients (Baselga et al. 2007).

Ertumaxomab is a drug with a dual mechanism of action. It is a monoclonal, trifunctional, bispecific antibody that with its two antigen-binding sites binds both HER-2 and CD3, which is a part of the T-cell receptor complex. Binding of the drug molecule forms a complex of T-cells, tumor cells and macrophages or dendritic cells. This leads to phagocytosis of tumor cells. Ertumaxomab is currently tested in phase II clinical trials (Bedard et al. 2009).

A novel irreversible HER-1 and HER-2 tyrosine kinase inhibitor, BIBW 2992 has shown promising anti-tumor activity in trastuzumab pre-treated patients in phase I and II clinical trials (Hickish et al. 2009). Neratinib is a tyrosine kinase inhibitor preventing signaling through all the HER family members and it has shown clinical activity as a single agent (Burstein et al. 2010) and is currently under investigation in combination with chemotherapeutic agents (Bedard et al. 2009).

Also several strategies fof anti-HER-2 vaccines have been developed. The vaccines can be based on whole cells, DNA, peptides, proteins or anti-idiotypic antibodies. The most advanced vaccines are those with peptides as antigens (Ladjemi et al. 2010). These vaccines have been assessed in patients with metastatic breast cancer to test whether they are able to induce prolonged immune responses. The phase I and phase II clinical trials have shown development of either new or improved immunity in patients with HER-2 positive breast cancer (Disis et al. 2009). In addition to developing new drugs, existing HER-2 targeted agents can be combined with other kinds of targeted therapy to improve response and patient survival. For instance, inhibitors of the mTOR (mammalian target of rapamycin) kinase, downstream in the PI3K pathway, are being studied in clinical trials in patients with HER-2 overexpressing breast cancer that has progressed on trastuzumab (Nahta et al. 2009).

5. Mechanisms of action of trastuzumab

Although trastuzumab has played an important role in the treatment of breast cancer for over ten years, the mechanisms of action are still unknown. Most important indicator for response to trastuzumab therapy is the HER-2 receptor status of the tumor (Vogel et al. 2002). Its importance has been shown also in tumor stem cells, which have elevated HER-2 expression and respond well

to trastuzumab treatment (Magnifico et al. 2009). In preclinical setting it has been shown that trastuzumab is able to block shedding of HER-2 receptors by metalloproteases. Thereby it would prevent formation of truncated p95 –forms, which remain bound to cell membrane and have constitutive tyrosine kinase activity (Molina et al. 2001). Existence of these proteolytically cleaved extracellular domains (ECD) in serum has been associated with progressive metastatic disease and reduced response to chemotherapy and endocrine therapy (Lipton et al. 2002, Colomer et al. 2004). Several studies have shown a correlation between serum ECD levels prior to treatment and response to trastuzumab as well as a decline in serum HER-2 levels during treatment (Esteva et al. 2002, Köstler et al. 2004, Esteva et al. 2005). This would point that patients with elevated ECD levels have higher response rates. It has been stated that binding of trastuzumab to serum ECD helps to clear them from circulation and thereby allows the remaining antibody to bind to available full-length HER-2 receptors on the cell surface. Most of these studies have been performed with patients receiving a combination of trastuzumab and chemotherapy. Thus, the declines in serum ECD may not be due to actions of trastuzumab alone. Because of the conflicting results obtained by other clinical studies (Burstein et al. 2003, Lennon et al. 2009) the role of ECD as a marker of trastuzumab response is still unclear. It has also been stated that serum ECD binds and neutralizes trastuzumab, which results to inability of the antibody to bind to available intact full-length receptors. Initially it was proposed that binding of trastuzumab to HER-2 leads to receptor internalization and endocytosis (Hudziak et al. 1989) but more recent studies have disproven this as a mechanism of action (Austin et al. 2004, Hommelgaard et al. 2004).

Antibody dependent cellular cytotoxicity (ADCC) has been suggested to have an essential status in the function of trastuzumab. Natural killer (NK) cells recognize the Fc part of trastuzumab molecules bound to HER-2 positive tumor cells. This evokes recruitment of immune effector cells, which attack the target tumor cells. Also intrinsically trastuzumab resistant cells show partial trastuzumab response as a result of ADCC activity (Barok et al. 2007). The significance of ADCC has been shown also in clinical setting. Certain inherited polymorphisms in the Fc receptors of the NK cells are associated with an improved response rate and progression free survival in patients with metastatic breast cancer treated with trastuzumab and taxane (Musolino et al. 2008). In another study patients with partial or complete response were reported to have a higher ADCC and a higher infiltration of NK cells (Gennari et al. 2004). This was observed also when trastuzumab was combined with docetaxel (Arnould et al. 2006).

HER-2 expression is linked with expression of multiple pro-angiogenic mediator proteins and trastuzumab thus reduces microvascular density, vascular permeability and endothelial migration (Izumi et al. 2002, Klos 2003, Wen et al. 2006) in tumor cells. Treatment with

trastuzumab also induces arrest in the G1 phase of the cell cycle resulting in reduction in proliferation in cancer cells (Sliwkowski et al. 1999).

One of the central mechanisms of action of trastuzumab is inhibition of the PI3K-PTEN signaling pathway. This is shown to occur by diverse mechanisms. Nagata et al. (2004) observed that trastuzumab leads to activation of the tumor suppressor PTEN in breast cancer cells thus reducing phosphorylated and activated AKT. Inhibition of PI3K activity was not detected in that study. In a more recent study Junttila et al. (2009) showed that trastuzumab treatment results in dephosphorylation of HER-3, which leads to dissociation of PI3K from the receptor. Trastuzumab is also shown to inhibit breast cancer stem cells through disturbing PI3K signaling (Korkaya et al. 2008). Mechanisms of action of trastuzumab are presented in Table 2.

6. Mechanisms of action of lapatinib

Lapatinib acts by binding to the intracellular tyrosine kinase domains of HER-1 and HER-2, which leads to inhibition of phosphorylation. This inhibition has been shown to block activation of MAPK and PI3K proteins in breast cancer cell lines, tumor xenografts and clinical breast cancer samples (Xia et al. 2002, Rusnak et al. 2001, Spector et al. 2005). Thus the PI3K-PTEN pathway is an essential mediator also for the tumor growth inhibiting effect of lapatinib. Additionally, lapatinib down-regulates survivin expression in breast cancer cell lines and clinical tumors treated with lapatinib (Xia et al. 2006). Survivin is a member of the inhibitor of apoptosis protein family. In breast cancer survivin expression is suggested to be an independent prognostic factor predicting poor clinical outcome (Tanaka et al. 2000). This down-regulation is also dependent on PI3K inhibition and it is considered as one of the most important biological effects of lapatinib (Xia et al. 2006). Although being a dual tyrosine kinase inhibitor, the effect of lapatinib is associated with HER-2 status of the cancer cells (Konecny et al. 2006). Also clinically it has been shown that HER-2 amplification and overexpression are predictors of lapatinib response (Cameron et al. 2008). Because lapatinib does not target the extracellular part of HER-2, it has been shown to be active also against tumor cells expressing the truncated p95 form of the receptor in preclinical models (Xia et al. 2004, Anido et al. 2006) and in patients with high serum HER-2 ECD levels (Cameron et al. 2008). Mechanisms of action of lapatinib are presented in Table 2.

Table 2. Mechanism of action of trastuzumab and lapatinib

Mechanisms of action	*Trastuzumab*	*Lapatinib*
Interference with PI3K-PTEN pathway	Yes	Yes
Induction of cell-cycle arrest	Yes	Yes
Induction of apoptosis	Yes	Yes
Impairment of extracellular domain cleavage	Yes	No
Causes decreased angiogenesis	Yes	No
Antibody-mediated cellular cytotoxicity	Yes	No

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7. Trastuzumab resistance

Resistance to HER-2 targeted therapy is an important subject in the treatment of breast cancer patients and therefore under continuous research. Research is directed also towards different ways to overcome resistance by e.g. completely new drugs or combinations of existing regimens with agents targeting other molecules in tumor cells. Although trastuzumab significantly improves the outcome for patients with HER-2 positive breast cancer, not all patients benefit from the therapy. A significant group of patients does not respond to the treatment at all (primary resistance), and most patients who initially respond to treatment generally tend to develop resistance during the treatment (acquired resistance). Majority of women develop resistance within one year of treatment initiation (Slamon et al. 2001).

Response to trastuzumab is shown to be strongly correlated with HER-2 amplification and overexpression of the tumor (Cobleigh et al. 1999, Vogel et al. 2002). Nevertheless, alterations in the ability of the antibody to bind HER-2 can result from different defects in the receptor and other proteins in the cell membrane. Trastuzumab is unable to bind the truncated p95 forms of the receptor lacking the extracellular domain (Xia et al. 2004, Anido et al. 2006). Expression of the p95 protein has been associated with trastuzumab resistance in both cell line studies and clinically (Scaltriti et al. 2007). MUC4 is a membrane associated mucin protein that contains EGF (epidermal growth factor) like domains. It protects cells from cellular and antibody mediated immune attacks and is often overexpressed in cancers. Cells that are completely resistant to trastuzumab are shown to express higher level of MUC4 and thereby have reduced ability to bind trastuzumab when compared with sensitive cell lines. Based on these results it is proposed that MUC4 blocks the binding of trastuzumab (Nagy et al. 2005). MUC4 is also an intramembrane activator of HER-2 (Ramsauer et al. 2003).

Blocking of HER-2 by trastuzumab can be overcome by increased signaling from other HER family receptors via ligand stimulation, which negates the inhibitory growth effects of

trastuzumab (Motoyama et al. 2002, Diermeier et al. 2005). Expression of HER-3 is shown to play an important role and to work cooperatively with IGF-1R (insulin-like growth factor receptor) and HER-2 in trastuzumab-resistant breast cancer cells. Formation of heterotrimers leads to activation of downstream signaling pathways and probably induces conformational changes in HER-2, which hinder trastuzumab binding (Huang et al. 2010).

IGF-1R, although not belonging to the HER receptor family has been demonstrated to have a role in trastuzumab resistance also independently. IGF-1R signaling and overexpression are shown to disturb the growth inhibitory effects of trastuzumab by antagonizing G1 –arrest (Lu et al. 2001, Lu et al. 2004). Dimerization of HER-2 with IGF-1R restores PI3K signaling in trastuzumab-resistant breast cancer cell lines while inhibition of IGF-1R induces apoptosis of tumor cells and inhibits HER-2 phosphorylation (Nahta et al. 2005). In clinical studies IGF-1R membrane expression is shown to associate with a lower response rate to trastuzumab and vinorelbine neoadjuvant therapy compared with tumors that express cytoplasmic IGF-1R (Harris et al. 2007). Another receptor tyrosine kinase outside the HER family, Met, is shown to be overexpressed in HER-2 positive breast cancer cell lines (Shattuck et al. 2008) and clinical tumors (Lindemann et al. 2007) and these two proteins act in synergism to promote cellular invasion (Khoury et al. 2005). *In vitro* Met is shown to contribute to trastuzumab resistance through sustained AKT activation (Shattuck et al. 2008).

Cell proliferation inhibiting effect of trastuzumab is partly due to induction of the cdk (cyclin-dependent kinase) inhibitor p27kip1 and increased formation of p27kip1-cdk2 complexes (Lane et al. 2000, Lane et al. 2001). *In vitro* it has been shown that trastuzumab resistant cells derived from an initially trastuzumab sensitive breast cancer cell line have decreased expression of p27kip1. Equally ectopic p27kip1 expression increases trastuzumab sensitivity in these cells. p27kip1 is a downstream effector of several signaling pathways, which may explain its role in trastuzumab resistance (Nahta et al. 2004b). Trastuzumab resistant cells are also shown to express higher levels of the cancer promoting survival factor Notch-1 protein a although HER-2 overexpressing cells in general have low Notch activity (Osipo et al. 2008).

Preclinical studies suggest that defects in the PI3K-PTEN pathway either by overactivated PI3K or loss of PTEN action can be associated with trastuzumab resistance (Nagata et al. 2004, Berns et al. 2007). Also several of the other proposed resistance mechanisms are related to aberrant PI3K-PTEN signaling. Trastuzumab is not able to block receptor dimerization (Nahta et al. 2004a) and the increased formation of heterodimers between HER-2 and other members of this receptor family can potentially lead to increased downstream AKT signaling and resistance to trastuzumab. The proposed resistance mechanisms of trastuzumab are presented in Table 3.

8. Lapatinib resistance

Resistance mechanisms of lapatinib are assumed to be different from those of trastuzumab. Lapatinib has not shown cross-resistance with trastuzumab in preclinical (Konecny et al. 2006, Nahta et al. 2007) or clinical studies (Geyer et al. 2006). Upregulation of PI3K signaling is proposed to be one of the mechanisms also behind lapatinib resistance, although opposing views have been presented. There are both preclinical (Xia et al. 2007, O'Brien et al. 2010) and clinical studies (Johnston et al. 2008) showing that loss of PTEN does not associate with lapatinib resistance. However, the results of a genome wide loss of function shRNA screen demonstrate PTEN loss to correlate with lapatinib resistance *in vitro*. This study also associated *PIK3CA* mutations with lapatinib resistance (Eichhorn et al. 2008).

Lapatinib downregulates AKT, which results in activation of the FOXO3a (forkhead box O3) transcription factor. FOXO3a promotes estrogen receptor signaling and further represses the apoptosis inhibitor survivin. This indicates that cancer cells resistant to lapatinib develop a compensatory pathway regulating cell survival. In preclinical studies cell lines resistant to lapatinib are shown to be viable despite effective inhibition of HER-2 and AKT (protein kinase B) functions. Treatment with combination of lapatinib and an estrogen receptor antagonist fulvestrant or complete estrogen deprivation induces apoptosis in these cells (Chen et al. 2008). In a clinical study the expression of FOXO3a in patients with early-stage breast cancer was shown to be increased in ER positive tumors during lapatinib treatment. The treatment also evoked localization of ER to the nucleus, which suggests activation of its transcription factor function (Xia et al. 2006). A novel mechanism for lapatinib resistance is increased expression of a membrane bound tyrosine kinase receptor AXL, which is associated also with resistance to other cancer therapies (Liu et al. 2009). The proposed resistance mechanisms of lapatinib are presented in Table 3.

Until now the studies have not been able to recognize any clinically verified biomarker that would predict response to trastuzumab or lapatinib. Identification of the factors behind the reduced effect of these drugs could reveal also molecular targets for overcoming resistance. Related to the proposed resistance mechanisms, examples of usable clinical tests could be *PIK3CA* mutation assays and protein assays detecting PTEN and HER-3. By recognizing the patients most likely to respond to therapy could result in avoidance of adverse side effects and remarkable medication costs. Also alternative treatment strategies could be implemented for potentially non-responsive patients.

Table 3. Proposed resistance mechanisms of trastuzumab and lapatinib

Resistance mechanism	*Trastuzumab*	*Lapatinib*
Altered target	Steric hindrance of receptor by cell surface proteins (MUC4) Truncated form of receptor (p95)	Probably not relevant
Alternative pathway signaling	IGF-1R overexpression	ER overexpression
Alternative HER signaling	HER1/HER3 heterodimers or HER1/HER1 homodimers Increased level of ligands (e.g. heregulin)	Probably not relevant
Constitutive activation of downstream effectors	Reduced level of PTEN and mutated PIK3CA Reduced p27kip1	Reduced level of PTEN and mutated PIK3CA, the results are contradictory

Modified from Nielsen et al. 2009

9. PI3K-PTEN –pathway

The PI3K-PTEN pathway is evolutionally conserved from yeast to mammals and it is required for many normal cellular functions, e.g. cell metabolism, polarity and vesicle trafficking (Engelman et al. 2006). PI3K-PTEN signaling also controls angiogenesis, growth, proliferation and senescence. In normal cells the function of this pathway is strictly controlled by upstream and downstream proteins but upregulation is a common event in several cancers and this cascade is of crucial importance in RTK (receptor tyrosine kinase) mediated oncogenic activity. Upregulation is also a way for tumor cells to prevent the growth inhibitory mechanisms of several anti-cancer therapies. Mechanisms of overactivation of this pathway include increased RTK signaling (by mutated HER-1, amplification of HER-2 or HER-3 amplification and overexpression of ligands), or genetic or epigenetic abnormalities in the signaling proteins of this pathway (Jia et al. 2009). PI3K-PTEN pathway is probably the most frequently disrupted pathway in human cancers and several kinds of targeted therapeutic agents against this pathway are under development.

9.1 PIK3CA oncogene

Class I phosphoinositide 3-kinase consists of two subunits; regulatory p85 and catalytic p110. The catalytic subunit occurs in four isoforms. All of the isoforms have oncogenic potential but mediate distinct signaling processes. The p110α (PIK3CA), encoded by the *PIK3CA* gene is the only isoform in which activating mutations have been reported (Kang et al. 2006). This subunit consists of five domains: an N-terminal adaptor-binding domain that binds to the p85-regulatory subunit, a

Ras-binding domain, a C2 domain, a helical domain, and a C-terminal kinase domain (Zhao et al. 2005, Vogt et al. 2007) (Figure 2). PI3K is activated by signaling from RTKs, which is followed by recruitment of the cytosolic protein to cell membrane. The regulatory subunit, p85, binds and integrates these signals and provides activation of p110 α (Stokoe et al. 1997).

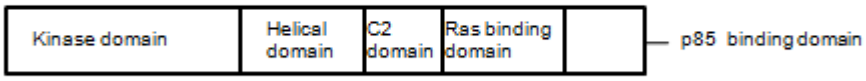

Figure 2.*The five domains of PIK3CA protein*

9.2 PTEN tumor suppressor gene

Also the *PTEN* gene is composed of five functional domains: a short N-terminal PIP2-binding domain, a phosphatase domain, a C2 domain, and a C-terminal tail containing PEST (proline, glutamic acid, serine, threonine) sequences and a postsynaptic density protein–*Drosophila* disc large tumor suppressor–zonula occludens 1 protein (PDZ)–interaction motif (Figure 3a). The phosphatase protein product of the gene, *PTEN* hydrolyzes PIP3 to PIP2, thereby acting as the catalytic antagonist of PI3K and negatively regulating AKT other pleckstrin homology domain-containing proteins that modulate cell growth, survival, and angiogenesis (Puc et al. 2005). The inactive conformation of PTEN is located in the cytosol and while activated, it moves to plasma membrane to bind PIP_2. PTEN has also protein phosphatase function with several targets. However, mutational studies have shown that disturbing the lipid phosphatase activity of PTEN results in inactivation of the tumor suppressor ability *in vitro* although the protein phosphatase activity is intact (Myers et al. 1998).

PTEN is shown to interact with the tumor suppressor p53 and it has been suggested that loss of PTEN function is associated with decreased p53 function. Also opposing results have been presented proposing that PTEN loss activates p53 (Chen et al. 2005). PTEN is shown to exist also as a nuclear protein and thereby control chromosomal stability and DNA repair (Shen et al. 2007). In all, PTEN has a vast amount of ways to maintain the normal function of cells.

9.3 PI3K-PTEN signaling

Signaling in the PI3K-PTEN pathway is presented in Figure 1. PI3K signaling is activated mainly by growth factor stimulation. The PI3K complex is recruited to the cell membrane either by direct interaction of the p85 subunit with the activated receptor tyrosine kinases or with adaptor proteins bound to receptor tyrosine kinases (Skolnik et al. 1991). Alternatively, the catalytic subunit of PI3K can be activated independently of the regulatory subunit by Ras (rat sarcoma) protein (Rodriguez-Viciana et al. 1994). The activated p110 catalytic subunit phosphorylates PIP2 (phosphatidylinositol 4,5 bisphosphate) at the 3 position of the inositol ring. The product, PIP3 (phosphatidylinositol 3,4,5-trisphosphate), provides a docking site for signaling proteins with pleckstrin-homology domains, among these PDK1 (phosphoinositide-dependent kinase 1) and AKT (Corvera and Czech 1998). PDK1 phosphorylates and thereby stimulates the catalytic activity of AKT (Alessi et al. 1997). Activated AKT phosphorylates up to 100 substrates, which results either in activating or inhibiting these downstream targets and further in cellular growth, survival, and proliferation (Carracedo and Pandolfi 2008). PTEN regulates the cellular levels of PIP3 by dephosphorylating PIP3 back to PIP2 (Maehama and Dixon 1998). In addition to receptor tyrosine kinases and PTEN, also several downstream proteins are shown to control PI3K activity (Carracedo and Pandolfi 2008).

10. PIK3CA and PTEN in breast cancer

There is genetic evidence that alterations in multiple components of the PI3K-PTEN pathway could be required in cancers. Although the *PIK3CA* mutations and loss of PTEN function both enhance PI3K signaling, these defects serve different functions. It has been suggested that they confer distinct selective advantages because e.g. in endometrial tumors *PTEN* mutations are found at similar frequencies in hyperplastic precursor lesions and carcinomas, while *PIK3CA* mutations selectively occur in carcinomas (Hayes et al. 2006). These events may, however, exist in different stages in different tumor types. A recent publication proposes that *PIK3CA* mutations are related to an early phase of breast cancer (Dunlap et al. 2010).

10.1 PIK3CA mutations and amplification

PIK3CA gene is often mutated in human cancers. Three major tumor types that show a high frequency of *PIK3CA* mutations are breast, endometrial and colorectal cancers. Mutation frequency in breast cancer is 16-40% in tumors and up to 50% in cell lines (Bachman et al. 2004, Campbell et al. 2004, Wu et al. 2005, Levine et al. 2005, Lee et al. 2005a, Saal et al. 2005, Hollestelle et al. 2007, Liedtke et al. 2008). The mutant form of p110α shows a gain of function in enzymatic and signaling activity and is shown to be oncogenic. *PIK3CA* mutated in one of the hot-spot locations has been shown to induce oncogenic transformation in chicken embryo fibroblasts (Kang et al. 2005) as well as in mammary epithelial cells (Isakoff et al. 2005, Zhao et al. 2005).

Mutations are almost exclusively missense mutations concentrated in hot spot regions in exons 9 and 20 (80-90% of mutations), corresponding to helical and tyrosine kinase domains, respectively. Thus most of the mutations affect residues that are highly conserved in evolution and are therefore likely of critical functional importance. This confirms the gain of function properties of the mutations (Samuels et al. 2004). In addition to these hot spot locations, mutations have been detected in almost all exons of the gene and most of them evoke increased lipid kinase activity (Kang et al. 2005, Gymnopoulos et al. 2007).

Results of structural studies have suggested a mechanism of p110α mutations for promoting cancer cell growth and survival. The wild type p110α subunit is stabilized by binding to the p85α subunit through its p85α domain and the negatively charged helical domain. RTK activation and subsequent phosphorylation recruits this complex to the membrane, which causes conformational changes leading to weakening of the interactions of the regulatory subunit on catalytic subunit and to high transitional p110α activity. In the presence of an exon 9 mutation, the negative charge of the helical domain is shown to convert to a positive charge, resulting in sustained p110α activity at the membrane and further delayed dissociation of the p85α subunit from the phospho-RTK (Huang et al. 2007).

Amplification of *PIK3CA* gene is a rare genetic alteration in breast cancer. There are two studies reporting increased gene copy number in clinical breast tumors (Wu et al. 2005, Kadota et al. 2009) and both gene amplification and mutations have also been observed in same tumors (Kadota et al. 2009). The relation of gene copy number and protein expression has not been studied in breast cancer. In ovarian cancers the amplification rate is higher and the increased gene copy number is associated with increased transcription, protein expression and kinase activity (Shayesteh et al. 1999). Mutations in the *PIK3R1* gene encoding the regulatory part p85α are observed in a variety of human cancers, including glioblastomas, ovarian cancers, and colorectal cancers (Philp et

al. 2001, Mizoguchi et al. 2004). Until now, these mutations have not been reported to occur in breast cancers.

There are several publications studying the association of *PIK3CA* mutations with clinicopathological characteristics of breast cancer. The mutations are shown to be more common in lobular cancer type (Buttitta et al. 2006) and in node negative tumors and tumors diagnosed at older age (Liedtke et al. 2008). Mutations are positively correlated with positive hormone receptor status (Stemke-Hale et al. 2008). An inverse correlation has been found between mutated *PIK3CA* and Ki-67 and HER-2 expression (Buttitta et al. 2006) while mutated tumors are also reported to be more often HER-2 positive (Stemke-Hale et al. 2008). Some publications have not been able to find correlation between mutated *PIK3CA* and other tumor characteristics, such as HER-2 status, patient age at diagnosis, tumor size, lymph node metastasis, histological grade, and ER and PR status (Buttitta et al. 2006, Santarpia et el. 2008). Mutations have been associated with both unfavorable (Li et al. 2006) and favorable outcome (Kalinsky et al. 2009), while some studies have not been able to find association with patient outcome (Santarpia et al. 2008). The results of different studies vary depending on the quantity and quality of study material and methods used. Also different tumor features have been associated with exon 9 and exon 20 mutations. *PIK3CA* mutations are rarely found in basal-like breast cancers (Stemke-Hale et al. 2008) but are reported to be frequent in metaplastic breast cancers, which are another aggressive type of triple negative cancers (Hennessy et al. 2009).

Activation of PIK3CA has been associated with resistance to several chemotherapeutic agents *in vitro* (Jin et al. 2003, Knuefermann et al. 2003) but not in clinical breast tumors (Liedtke et al. 2008). However, mutated *PIK3CA* is shown to be negatively correlated with response to endocrine therapy (Ellis et al. 2010).

10.2 Loss of PTEN

PTEN is one of the most frequently mutated, deleted and silenced tumor suppressor genes in human cancers (Keniry and Parsons 2008). Impaired PTEN function leads to PIP_3 accumulation in cells and to an unrestrained activation of its downstream signals, e.g. AKT signaling (Maehama and Dixon 1998, Chow et al. 2006). PTEN loss has also been shown to upregulate kinase signaling upstream of PI3K (Miller et al. 2009). Respectively, overexpression of wild type PTEN in cancer cells is shown to induce apoptosis and block cell-cycle progression, colony formation and cell migration (Li and Sun 1998, Tamura et al. 1998, Weng et al. 2001). Defects in PTEN are proposed to be more transforming events than *PIK3CA* mutations (Miller et al. 2009). It has also been shown

that loss of PTEN leads to different cellular changes than *PIK3CA* mutations (Blanco-Aparicio et al. 2007). Co-existence of these defects is supported also by the result that combining *PIK3CA* mutation with knock-down of PTEN remarkably increased AKT phosphorylation in tumor cells (Oda et al. 2008). There is also other evidence showing that these defects result in different signaling outputs. Miller et al. (2009) have shown that knockdown of *PTEN* in breast cancer cell lines with activating *PIK3CA* mutations results in hormone-independent growth. More importantly, loss of PTEN enhanced IGF-1R/IRS-1 and HER3 signaling. Nevertheless, PTEN loss and *PIK3CA* mutations have been reported to be mutually exclusive *in vitro* (Oda et al. 2008) and *in vivo* (Saal et al. 2005).

PTEN loss can occur via several mechanisms (Figure 3). The mutation frequency of *PTEN* in breast cancer is low, only 3.5 % on average (Yin and Shen 2008) but loss of expression is remarkably more common, in 20-25% of breast cancers (Fujita et al. 2006, Nagata et al. 2004, Saal et al. 2005, Berns et al. 2007). Incidence of loss of heterozygosity (LOH) is reported to be 40% in breast cancers, which is much more frequent than biallelic inactivations (Singh et al. 1998, Feilotter et al. 1999). Studies with mouse models propose that *PTEN* is functionally haploinsufficient, which means that one functional allele is not enough to retain a wild-type condition (Di Cristofano et al. 2001). A recent publication using mouse models has shown that even a minor decrease in *PTEN* expression is sufficient to promote cancer susceptibility (Alimonti et al. 2010). Complete loss of PTEN is more frequent in metastatic cancer than primary tumors (Di Cristofano and Pandolfi 2000).

Inactivating genetic alterations of *PTEN* include different types of point mutations and deletions of the whole gene or exons 6-9. Both somatic and germ line *PTEN* mutations are found in cancers. Inherited PTEN mutations result in wide variation of familial syndromes with cancer disposition and multiple benign tumors (hamartomas), as well as a risk for several carcinomas. These syndromes include Cowden syndrome (CS), Bannayan–Riley–Ruvalcaba syndrome (BRRS), Proteus syndrome (PS), and Proteus-like syndrome (PSL). They are clinically distinct, representing a highly variable spectrum of abnormalities, such as thyroid abnormalities and fibrocystic disease (CS), macrocephaly, lipomatosis, hemangiomatosis and speckled penis (BRRS), lipomas and overgrowth (Eng et al. 2003, Zbuk and Eng 2007). Although rare in breast cancer, mutations occur in almost all domains of *PTEN* (Figure 3a). This suggests that all the domains are physiologically relevant to tumorigenesis (Catalogue of Somatic Mutations in Cancer). Most of the missense mutations found in cancer, however, have been detected in the phosphatase domain thereby affecting the catalytic activity of PTEN. This emphasizes the importance of phosphatase activity in tumor suppression (Eng 2003). Also nonsense and frameshift mutations leading to the formation of

truncated PTEN protein lacking the C-terminal tail and the PDZ-interaction motif are shown to fail to suppress PI3K signaling *in vitro* (Georgescu et al. 2000, Vazquez et al. 2006).

PTEN gene transcription can be decreased by promoter silencing by DNA methylation (Mirmohammadsadegh et al. 2006, Wiencke et al. 2007). Methylation has been observed both in early and in situ (Muggerud et al. 2010) as well as invasive (Khan et al. 2004) breast cancers. MicroRNAs (miR) are small, endogenous, non protein coding RNAs that regulate gene expression posttranscriptionally (Bartel 2004). MicroRNAs are shown to have an important role in tumorigenesis. MiR-21 is upregulated in several tumor types targeting tumor suppressor genes, also *PTEN*. Expression or miR-21 has been statistically significantly associated with low PTEN in clinical breast tumor samples (Huang et al. 2008). Mechanisms of posttranslational modifications include acetylation, phosphorylation and ubiquitinylation (Figure 3b). Phosphorylation of the C – terminus stabilizes PTEN but reduces its activity toward PIP_3. Acetylation regulates PTEN negatively by interfering with its catalytic specificity toward PIP_3. Reactive oxygen species oxidize certain residues in the catalytic domain of PTEN, resulting in its inactivation (Seo et al. 2005). Ubiquitination of PTEN controls its nuclear localization and stability (Maccario et al. 2010). Although there are a multitude of posttranslational mechanisms that can modulate PTEN activity, it is a relatively stable protein. The functional relevance of these regulatory mechanisms in tumorigenesis remains to be unclear.

Knock down of *PTEN* in breast cancer cells is shown to result in antiestrogen resistance (Miller et al. 2009). *In vitro* studies have also shown that both lipid phosphatase activity and protein phosphatase activity are important in determining the sensitivity of breast cancer cells to chemotherapeutic drugs. Introduction of phosphatase deficient PTEN mutants into MCF-7 cells decreased the sensitivity to doxorubicin. PTEN mutant lacking both lipid and protein phosphatase activity is shown to confer more resistance to cancer chemotherapeutic doxorubicin than the mutant lacking only lipid phosphatase activity (Steelman et al. 2008).

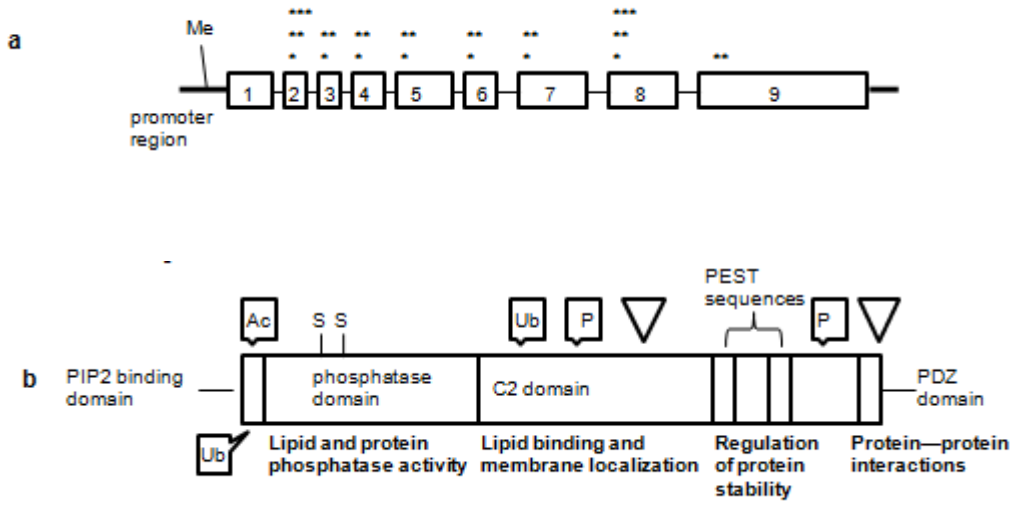

Figure 3. *Different mechanism of PTEN loss*
a) Occurrence of different types of mutations in exons of PTEN. In addition to mutations, also methylation (Me) of the promoter area can recude PTEN expression.
** substitution (missense or nonsense), ** deletion, *** insertion*
b) Inactivating post-translational modifications of the PTEN protein
Ac = acetylation, Ub = ubiquitination, S = disulfide formed during oxidation,
P = phosphorylation, triangle = proteolytic cleavage

10.3 Aberrant PIK3CA and PTEN in HER-2 positive breast cancer

PIK3CA mutations are reported to associate with HER-2 protein overexpression (Oda et al. 2008) as well as gene amplification (Brachmann et al. 2009) in breast cancer cell lines. Similar results have been published in some studies with clinical tumor samples (Saal et al. 2005, Stemke-Hale et al. 2008), while also opposite results have been presented (Buttitta et al. 2006).

Reduced PTEN expression has been shown to be inversely correlated with HER-2 overexpression in breast cancer cell lines (Oda et al. 2008) and clinical tumor samples (Marty et al. 2008). There is also preclinical (Brachmann et al. 2009) and clinical evidence connecting PTEN loss with the triple negative tumor type (Marty et al. 2008, Song et al. 2010). Defects in the PI3K-PTEN signaling pathway have been associated with resistance to HER-2 trageted drugs in several studies. Introducing PTEN defects in trastuzumab sensitive breast cancer cells is shown to lead to resistance to trastuzumab as a single agent and also when combined with paclitaxel chemotherapy. Xenografts with defective PTEN did not either respond to trastuzumab treatment. Combination with PI3K inhibitors enhanced effect of trastuzumab *in vitro* and *in vivo* (Nagata et al. 2004). Trastuzumab response has been associated with the level of PTEN protein expression also in clinical trials. When patients with HER-2 positive metastatic breast cancer were treated with

combination of trastuzumab and chemotherapy, those with PTEN protein deficiency had significantly lower response when compared with PTEN positive tumors. In the control group of HER-2 negative patients PTEN expression did not affect response to chemotherapy (Nagata et al. 2004). Berns et al. (2007) have proposed that existence of aberrant PTEN status and *PIK3CA* mutations together is a statistically significant biomarker for prognosis after trastuzumab therapy.

AIMS OF THE STUDY

The aims of the present study were

- To study the incidence trends of HER-2 positive breast cancer in a defined area in Finland
- To study the intrinsic trastuzumab resistance mechanisms *in vitro*
- To analyze *PIK3CA* mutations and lack of PI3K expression in breast cancer cell lines and clinical breast tumor samples
- To study PTEN expression on mRNA and protein level in breast cancer cell lines and clinical breast tumor samples
- To assess the clinicopathological characteristics and breast cancer specific survival of breast cancer patients with aberrant PI3K-PTEN signaling

MATERIALS AND METHODS

1. Tumor samples and cell lines

1.1 Study I

The study material consists of three population-based cohorts of breast cancer patients, diagnosed during the years 1982 to 1986 (n = 310), 1989 to 1992 (n = 108), and 2004 to 2005 (n = 713) in the Pirkanmaa Hospital District, Finland. All patients in the study had primary invasive breast cancer confirmed histopathologically. The patients diagnosed in 1982 to 1986 and 1989 to 1992 were selected randomly from the Finnish Cancer Registry database, which achieves close to 100% completeness (Teppo et al. 1994). Axillary lymph node status data were derived from the Finnish Cancer Registry, which was used to ascertain the similarity of the cohorts. The cohorts comprised 32.4% and 11.0%, respectively, of all invasive breast cancer patients reported to the Cancer Registry from the study area. The newest cohort (cancers diagnosed between January 2004 and December 2005) included all invasive breast cancers submitted for hormone receptor and HER-2 analysis. This cohort consisted of 91.8% of cases reported to the Finnish Cancer Registry for this district.

1.2 Study II

The human breast cancer cell line EFM-192A was obtained from the German Resource Center for Biological Material and the cell lines BT-474, MDA-MB-361, MDA-MB-453, MCF-7, SK-BR-3, UACC-812, UACC-893 and ZR-75-30 were obtained from the American Type Tissue Culture Collection. The JIMT-1 cell line has been established in the laboratory of Cancer Biology, University of Tampere, Finland (Tanner et al. 2004) (cell line also available via German Resource Center for Biological Material). The cell lines were grown under conditions recommended for each line.

1.3 Study III

Samples of 256 fresh-frozen invasive primary breast tumors were studied for *PIK3CA* mutations. RNA from 138 samples was available for qRT-PCR analysis of PTEN. Routine formalin-fixed and paraffin-embedded samples from 45 tumors with *PIK3CA* mutation and additionally 78 *PIK3CA*

wild type tumors were analyzed by immunohistochemistry. Samples were derived from the Institute of Medical Technology, University of Tampere and from the archives of the Department of Pathology at Tampere University Hospital, with the permission from National Supervisory Authority for Welfare and Health. Survival rates of all patients were calculated by the method of Kaplan and Meier. Survival data collected from the death certificates was obtained from the Finnish Cancer Registry with the permission from National Institute for Health and Welfare.

2. In situ hybridization (I, III)

Formalin-fixed and paraffin-embedded tumor samples were used for CISH and FISH. Amplification of HER-2 was determined in all tumors as previously described (Isola et al. 2004). The cut-off for amplification was set at six gene copies per cell or the presence of a typical gene copy cluster (studies I, III). Cell line gene copy numbers were confirmed by using the dual-color fluorescence in situ hybridization (FISH;HER-2 and 17 centromere). After trypsinization and washing with PBS, the cells were fixed with methanol-acetic acid (3:1) and air-dried on slides. A bacterial artificial chromosome (BAC) clone specific to HER-2 DNA was labeled with dUTP-FITC (Fermentas, Burlington, Canada) and the chromosome 17 centromere probe (p17H8) was labeled with chromatide Alexa Fluor 594-5-dUTP (Invitrogen, Carlsbad, CA) using nick-translation and FISH was performed according to an established technique (study II).

3. PIK3CA mutation analysis by DHPLC (II, III)

The samples were investigated for mutations in exons 6, 7, 9, 14 and 20 in the PIK3CA gene in study II. These exons were selected on the basis of previous studies in which breast cancers were screened for mutations in all 20 exons and mutations were identified in the five aforementioned exons (Campbell et al. 2004). Based on the results of the study with cell lines, the clinical tumor samples were screened for mutations in exons 6, 7, 9 and 20. DNA was extracted and purified from trypsinized cells using a QIAamp Mini Kit (Qiagen, Hilden, Germany). The DHPLC (denaturing high performance liquid chromatography procedure was carried out as described elsewhere (Korja et al. 2005). The forward and reverse oligonucleotide primers used to amplify the *PI3KCA* exons are presented in the Table 4. The samples with abnormal-like elution profiles (showing a peak with a shoulder) in DHPLC were subjected to automated sequencing as described (Korja et al. 2005).

DHPLC was selected for the method due to its excellent sensitivity (Xiao and Oefner 2001) and its effectiveness in screening large amount of samples. Sequencing is considered as the

gold standard of mutation analysis and was therefore selected to confirm and determine the position of mutations.

Table 4. *The PCR primers for PIK3CA exons 6, 7, 9, and 20.*

Exon	*Primer sequences (5'→3')*
6	TTGGTTGATCTTTGTCTTCGTG
	TCAATCAGCGGTATAATCAGGA
7	GGGGAAGAAAAGTGTTTTGA
	AGACCAATTGCCATGCTCTT
9	GATTGGTTCTTTCCTGTCTCTG
	TGCTGAGATCAGCCAAATTC
20	CATTTGCTCCAAACTGACCA
	GGTCTTTGCCTGCTGAGAGT

4. Quantitative real-time RT-PCR (II, III)

Total RNA from the cell lines and tumor specimens was isolated using the Sigma GenElute Mammalian Total RNA Kit (Sigma–Aldrich, St Louis, MO) and the RNA samples were used for the first strand cDNA synthesis with Superscript II reverse transcriptase and random hexamer primer (Invitrogen, Carlsbad, CA). Quantitative real-time RT-PCR for NRG1 was performed using the LC-FastStart DNA Hybridization Probes Kit (Roche Diagnostics, Mannheim, Germany) (Staff et al. 2003) with TBP as reference gene (study II). PTEN cDNA was amplified using the Dynamo SYBR Green Kit (Finnzymes Oy, Espoo, Finland) according to the manufacturer's instructions. cDNA quantities were analyzed in duplicate, normalized against GAPDH as an internal control gene and expressed in relation to cDNA from MCF-7 cell line with a known normal PTEN gene status (DeGraffenried et al. 2004) (studies II, III). The results are expressed as the relative gene expression using the ΔΔCp method. The primer and probe sequences are presented in Table 5.

Table 5. *The real-time RT-PCR primer and probe sequences for the PTEN, GAPDH, Neuregulin 1 and TBP genes.*

Gene	*Primer sequences (5'→3')*	*Hybridization probe sequences (5'→3')*
PTEN	ATTGCAGAGTTGCACAATATCC CACATATCATTACACCAGTTCGTC	
GAPDH	CTGAGCTAGACGGGAAGCTC TCTGAGTGTGGCAGGGACT	
NRG 1	ATCCACAGAAGGAGCAAATA CACTTGCACAAGTATCTCGA	CTTCTCCGCACATTTTACAAGATG-Fluorescein Red640-CTTGTCCCAGTGGTGGATGTAGA
TBP	GAATATAATCCCGGCTTTGT ACTTCACATCACAGCTCCCC	TTTCCCAGAACTGAAAATCAGTGC-Fluorescein Red640-TGGTTCGTGGCTCTCTTATCCTCATG

5. Measurement of antibody-dependent cellular cytotoxicity (II)

ADCC was analyzed by measuring the lactate dehydrogenase (LDH) released from the breast cancer cells as a result of ADCC activity of peripheral blood mononuclear cells (PBMC). The PBMCs were separated from the heparinized blood of a single healthy donor by Ficoll density gradient centrifugation (Histopaque-1077, Sigma–Aldrich, St. Louis, MO). Breast cancer cells (target; 5000–10,000 per well) and PBMCs (effector) were co-incubated at a ratio of 1:40 (target:effector) in 100 µL of DMEM containing 5% FBS. This coincubation was performed in a 96-well U-bottomed plate in quadruplicate for 6 h at 37 °C with trastuzumab or the negative control antibody, rituximab. ADCC was measured in a 6-h LDH release assay (CytoTox Non-Radioactive Cytotoxicity Assay, Promega Corporation, Madison, WI). The absorbance at 490 nm was recorded by using a microplate reader (Model 680XR, Bio-Rad, Hercules, CA). The negative control sample (target spontaneous) was prepared identically, but did not contain PBMCs; the effector spontaneous sample contained no target cells. Tumor cells killed by freezing at -80 °C for 1 h and then warmed up to 37 °C served as positive control (target maximum). The percentage of cells killed was calculated according to the following formula: (experimental - effector spontaneous - target spontaneous)/(target maximum - target spontaneous) x 100.

6. Measurement of trastuzumab binding capacity (II)

For flow cytometric measurements trypsinized cells were centrifuged and resuspended in 50 µl PBS (pH 7.4) supplemented with 1% BSA and labelled with a saturating concentration (40 µg/mL) of unlabeled trastuzumab on ice for 30 min. After labeling, the cells were washed twice with PBS and labelled with FITC-GAHIG (goat-anti human IgG (H + L), Jackson ImmunoResearch Europe Ltd.,

Newmarket, Suffolk, UK) on ice for 30 min. Next, the cells were washed twice with PBS and fixed in 1% formaldehyde. The fluorescence intensity of FITC was determined by using a Coulter1 EPICS XL-MCL flow cytometer (Beckman Coulter, Inc., Fullerton, CA).

7. *In vitro* assay of drug sensitivity (II)

The effect of trastuzumab and lapatinib on the growth of the HER-2 positive cells was examined by the AlamarBlue method (Invitrogen, Carlsbad, CA) with the HER-2 negative MCF-7 cell line as a control. The cells were trypsinized and plated at suitable densities in 96-well, flat-bottomed, tissue culture plates. The effect of trastuzumab was tested at concentrations of 0.1, 1, and 10 µg/mL and that of lapatinib at 0.1, 1, and 10 µmol/L. We determined the number of viable cells at 72 h after drug addition by adding the AlamarBlue reagent. The fluorescence was measured after a 12 h incubation period according to the manufacturer's instructions, with excitation occurring at 544 nm and emission at 590 nm using a Wallac Victor2 plate reader (Perkin–Elmer, Turku, Finland). Fluorescence values of samples were normalized with values of the culture media without cells. The results presented as the proportion of viable cells were calculated by dividing the fluorescence values of drug treated samples by the fluorescence values of untreated control samples.

8. Immunohistochemistry (III)

For immunohistochemical (IHC) staining the sample slides were first deparaffinized and rehydrated and subsequently pre-treated. Pre-treatment for PIK3CA, K5/14 antibody cocktail, and PTEN antibodies was carried out at 98 °C for 15 min and for ER, PR, and Ki-67 antibodies at 98 °C for 45 min with 0.05 M Tris-HCl buffer, pH 9.0 containing 0.001 M EDTA. Immunostainings were carried out with an Autostainer 480 (LabVision, Fremont, CA, US) automated immunostainer, the primary antibodies were incubated for 30 min. Information on the primary antibodies used in this study is presented in Table 6. A Powervision+ polymer kit (Leica Biosystems Newcastle Ltd., Newcastle, UK) was used for detection. The reactions were visualized with diaminobenzidine (DAB) chromogen (5 min, ImmPACT DAB, Vector Laboratories Inc., Burlingame, CA, US). Hematoxylin (Mayer's hematoxylin, Oy FF-Chemicals Ab, Haukipudas, Finland) was used as a counter stain. PIK3CA antibody specificity was validated by using MCF-7 and SKBR-3 cell lines (obtained from the American Type Culture Collection, Rockville, MD, USA) as controls and PTEN antibody by staining 20 randomly selected samples with another antibody (Monoclonal Anti-Human PTEN (clone 6H2.1), Cascade Bioscience, Winchester, MA, USA).

For ER and PR samples with labeling index >10% were determined as positive. The Ki-67 immunostained samples were analyzed with ImmunoRatio software, which was calibrated to match visual cell counting (Tuominen et al. 2010). PIK3CA-stained samples were scanned using ScanScope XT (Aperio Technologies Inc., Vista, CA, US) and any clearly distinguishable immunoreaction (brown DAB precipitate) was interpreted as positive. Selected three image areas were analyzed and averaged with ImmunoRatio, which was used to calculate the percent of DAB stained area out of the hematoxylin stained area. Staining of ER, PR, PTEN, and K5/14 were evaluated with a conventional light microscope.

Table 6. *The primary antibodies used*

Antibody	*Clone*	*Distributor*	*Dilution*
CK5	XM26	Novocastra	1:100
CK14	LL002	Novocastra	1:100
PIK3CA	polyclonal	Sigma-Aldrich	1:300
PTEN	138G6	Cell Signaling	1:100
PTEN control	6H2.1	Cascade Bioscience	1:100
Ki-67	MM1	Novocastra	1:500
ER	6F11	Novocastra	1:600
PR	16	Novocastra	1:500

9. Statistical methods (I-III)

Time trends in the proportion and incidence of HER-2-positive tumors were analyzed by regression methods, using a generalized linear model with binomial distribution and logarithmic link function and incidence trends with Poisson regression, both in Stata 8.0 (StataCorp, College Station, Tx, USA). The outcome was a HER-2-positive tumor and the explanatory variable was the year of diagnosis as continuous variable. The exponentiated regression coefficient indicates average change in the proportion of HER-2-positive tumors per year relative to the first year analyzed. Statistical significance was assessed using a likelihood ratio test.

Fisher's exact test and Chi-square test were used to test the significance of the cross-tabulated data. The comparisons between HER-2 gene copy number and expression of trastuzumab binding capacity, PTEN expression levels and PIK3CA mutation status of the cell lines, *in vitro* growth-inhibitory effects of trastuzumab and lapatinib, *PIK3CA* mutation status and drug response in cell lines, trastuzumab binding capacities of the breast cancer cells and the trastuzumab-mediated ADCC activity. Concordance between PTEN IHC and qRT-PCR findings in clinical breast tumor samples was estimated using the kappa test (using SPSS 16.0 [SPSS Inc., Chicago, IL). Survival

analyses were calculated using Kaplan-Meier life table curves. Breast cancer specific survival was calculated from the primary diagnosis to the date of death from breast cancer using GraphPad Prism 4 (GraphPad Software Inc., La Jolla, CA).

RESULTS

1. Incidence of HER-2 positive breast cancer (I)

In study I, changes in the proportion and incidence of HER-2 positive cancer were studied in three cohorts of breast cancers during years 1982-2005. In the earliest cohort the proportion of breast cancers with HER-2 amplification was 21.6% while in the latest cohort it had declined to 13.6%. These figures were then used to estimate the incidence trends of HER-2-positive breast cancer in the population. The average age-adjusted incidence of all invasive breast cancers was found to have increased by 40% during the study period (from 56.3/100,000 to 95.3/100,000), which is very close to the figures reported for the whole country (Finnish Cancer Registry). For the HER-2 positive breast cancer the trend was nearly steady. The age-standardized incidence had increased only slightly, from 12.2/100,000 to 13.0/100,000 (age adjusted to WHO standard population). In contrast, when multiplying the incidence of breast cancer with the proportion of HER-2 negative tumors (78.4%, and 86.4%, respectively), we found that the incidence of HER-2 negative cancer had almost doubled (from 44.1/100,000 to 82.3/100,000 women). The Poisson regression analysis indicated a 2% annual increase in HER-2-negative cancer (incidence rate ratio = 1.021, 95% confidence interval (CI) = 1.016 to 1.026). For HER-2-positive cancer there was no trend (incidence rate ratio = 1.000, 95% CI = 0.989 to 1.012). Adjusting the results according to the age of the patients assayed for HER-2 did not affect the results.

To find out whether the early diagnostics would affect the results, a more detailed analysis was performed for the most recent cohort. We found that 33.4% of all invasive tumors were detected by screening mammography among the screened age groups. The HER-2 positive, estrogen receptor and progesterone receptor negative, or triple-negative tumors were statistically significantly underrepresented in patients whose cancers were detected by mammography screening ($P = 0.039$, $P < 0.0001$, $P < 0.0001$, and $P = 0.100$, respectively).

The results of this study showed that the incidence of HER-2 positive breast cancer is lower than previously has been estimated and it has remained almost stable during the study period. Simultaneously the incidence of HER-2 negative breast cancer has increased remarkably.

2. Drug resistance in HER-2 positive breast cancer cell lines (II)

2.1 In vitro sensitivity of HER-2 positive cell lines to trastuzumab and lapatinib

In this study the AlamarBlue method was used to determine the sensitivity of breast cancer cell lines to trastuzumab and lapatinib. Our panel of cell lines consisted of the trastuzumab resistant JIMT-1 cell line, eight other HER-2 positive cell lines previously reported to be trastuzumab sensitive and the HER-2 negative MCF-7 cell line as a negative control. Trastuzumab had a moderate growth inhibiting effect on most of the HER-2 amplified cell lines. Lapatinib produced a more significant action and the IC50 effect, indicating the concentration required for 50% inhibition of the cell growth, was achieved with all HER-2 positive cell lines. Trastuzumab showed no growth-inhibitory effect on the JIMT-1 or MCF-7 cell lines when compared with the untreated controls. Evident inhibition in the growth of the most trastuzumab sensitive BT-474 cells was observed even at the lowest concentration. BT-474 cells were also more sensitive to lapatinib than the JIMT-1 cells. The trastuzumab and lapatinib responses of the HER-2 positive cell lines were compared by expressing the results in relation to the BT-474. A positive but statistically nonsignificant correlation between the growth-inhibitory effects of these two agents was detected ($r = 0.64$, $p = 0.06$). The JIMT-1 cell line was found to have the greatest resistance to the both drugs.

2.2 Trastuzumab binding capacity

The next objective was to find out the implication of different resistance mechanisms in the cell lines studied. The trastuzumab binding capacity reflects the expression of HER-2 protein as well as the effect of factors that may modulate trastuzumab binding, such as masking by MUC4 (Nagy et al. 2005). The HER-2 amplified cell lines showed a wide variation in the receptor expressions. JIMT-1 had the lowest levels of trastuzumab binding HER-2 receptors and the second-lowest expression was approximately 2-fold higher than that observed in JIMT-1 while the highest expression was approximately 8-fold higher than JIMT-1. HER-2 nonamplified MCF-7 cells also showed low levels of trastuzumab binding HER-2 protein.

The trastuzumab binding capacities of the cell lines were compared with the growth-inhibitory effect of both drugs and the results showed that the JIMT-1 cell line with the lowest trastuzumab binding capacity was also among the most trastuzumab-resistant cell lines. Respectively, BT-474 as the most trastuzumab-sensitive, expressed trastuzumab binding receptors many-fold compared to the two afore mentioned cell lines. However, this connection between

receptor expression and drug response was not observed in all HER-2 positive cell lines. These results point out that despite high trastuzumab binding capacity, the cell may have other defects that prevent trastuzumab activity.

2.3 Trastuzumab-mediated antibody-dependent cellular cytotoxicity

Trastuzumab-mediated ADCC is a well-recognized mechanism of trastuzumab action. Therefore we wanted to find out whether there are differences in the ADCC reaction between the HER-2 positive breast cancer cell lines or whether the trastuzumab binding capacity of the HER-2 receptors correlates with the strength of ADCC reaction as could be expected. However, our results showed that the HER-2 positive cell lines exhibited very low variation in terms of the fraction of cells killed by the ADCC reaction. MCF-7 cells with no HER-2 gene amplification and very weak trastuzumab binding capacity exhibited low levels of ADCC-mediated killing. All HER-2 overexpressing breast cancer cell lines exhibited significantly higher ADCC-mediated cell lyses. Although expressing lowest levels of HER-2, the JIMT-1 cells displayed remarkable ADCC activity. According to our results the trastuzumab binding capacities of the breast cancer cells did not correlate with the trastuzumab-mediated ADCC activity ($r = 0.4$, p = N.S.). This result was surprising, since ADCC reaction is initiated by binding of trastuzumab. However, there is currently no known biomarker for intensity of ADCC reaction.

2.4 NRG1 mRNA expression

The endogenous ligand for HER-3 receptor, NRG1, is shown to induce trastuzumab resistance in HER-2 positive cell lines. Therefore the relative level of NRG1 expression was examined in nine of the 10 studied cell lines. A detectable level of NRG1 mRNA was observed in two of the HER-2 positive cell lines. The expression of NRG1 mRNA in the JIMT-1 cell line was found to be 20 times greater than in the other NRG1 expressing cell line.

Taken together, these results indicate that drug resistance is a complicated phenomenon resulting from different biological properties.

3. PIK3CA in breast cancer cell lines and clinical tumor samples (II, III)

3.1 PIK3CA mutation status (II,III)

Mutation status of the *PIK3CA* gene was studied first in breast cancer cell lines. Mutations in the *PIK3CA* gene were found in five out of nine HER-2 positive breast cancer cell lines as well as in the HER-2 negative MCF-7 cell line. Most mutations were activating mutations located in the known hot spot regions in the exons 9 and 20 and two mutations were located in exon 7. All detected mutations were single nucleotide changes. The *PIK3CA* mutation status did not correlate with the trastuzumab sensitivities of the cell lines studied. Instead, *PIK3CA* mutations appeared to be related to lapatinib response.

In study III, *PIK3CA* mutation status was obtained from 244 clinical breast tumor samples. Mutations were found in 29.5% of tumors and all were missense mutations resulting in single amino acid substitutions. Also in the clinical tumors most of the mutations were found in the known hot spots in exons 9 and 20 and no mutations were found in exon 6. Four of the detected mutations occurred only once and no tumors harboured multiple mutations. All except one of the mutations have been (A1627G) reported to occur in breast cancers (Catalogue of Somatic Mutations in Cancer, Pérez-Tenorio et al. 2007, Michelucci et al. 2009). According to previous publications the A1627G (I543V) in exon 9 has been found in lymphoma (Abubaker et al. 2007). No tumors harbored multiple mutations. All mutations were missense mutations resulting in single amino acid substitutions.

3.2 PIK3CA protein expression (III)

Also PIK3CA expression was evaluated and correlated with tumor status in 42 mutation positive and 72 wild type tumors. The average PIK3CA protein expression, defined as the median of the percentage of positively stained cells, was nearly equal in tumors with mutation (32%) and in wild type tumors (29.5%). Tumors were classified into three groups according arbitrary cut points at 20% and 40% based on intensity and distribution of staining. This grouping showed that over third (35.7%) of the tumors with mutation expressed strong PIK3CA staining, while the proportion of wild type tumors in this group was only 22.2%. When the samples were classified according to the location of mutations, a larger proportion of positively stained cells was detected in samples with mutation in exon 20 in contrast to tumors mutated in exon 9 or wild type tumors.

3.3 Clinicopathological characteristics of PIK3CA mutation positive tumors (III)

Results of the mutation analysis were correlated with the tumor characteristics. In this study material we found mutated *PIK3CA* to be associated with positive hormone receptor (p = 0.0002) and negative HER-2 (p = 0.004) status. In line with this finding, *PIK3CA* mutations correlated inversely with the triple negative tumor type (p = 0.04). *PIK3CA* mutations were not associated with axillary lymph node status (p = NS) or age at diagnosis (p = NS). The basal-like (keratin 5/14 positive) phenotype did not either correlate with mutation status (p = NS). *PIK3CA* mutation was detected in 41.7% of samples with negative PTEN IHC staining result and 36.7 % of those stained positively for PTEN. Although *PIK3CA* mutations are known to activate AKT and thereby presumed to increase cell proliferation, the level of Ki-67 expression was not statistically significantly associated with mutation status of the tumors.

3.4 *Outcome in patients with PIK3CA mutation positive tumors (III)*

To find out whether the mutation status correlates with survival of the patients, a Kaplan-Meier analysis was performed with follow-up up to 20 years. During the first ten years the breast cancer specific survival of *PIK3CA* mutation positive and mutation negative patient groups was nearly identical. However, after ten years the survival for *PIK3CA* mutation positive tumors was poorer when compared with wild type tumors (p = 0.04).

Since *PIK3CA* mutations were associated with positive ER status, we analyzed patient survival separately in this subgroup. The positive ER status alone was associated with favorable outcome in the beginning of the follow-up irrespective of mutation status. During the last years of follow-up the survival groups of ER positive and ER negative tumor groups merged. When we divided the ER positive tumors into two groups according to *PIK3CA* mutation status we detected that the survival for ER positive and mutation positive tumors was poorer during the last ten years of follow-up when compared with the wild type tumors.

Among HER-2 positive cancers the survival for *PIK3CA* mutation positive tumors was poorer than for wild type tumors already from the beginning of the follow-up period (p = 0.04).

Our results are in line with the opinion that *PIK3CA* mutations are common and clinically important findings in breast cancer. We also found that *PIK3CA* mutations may be associated with late breast cancer specific mortality.

4. PTEN expression in breast cancer cell lines and clinical tumor samples (II, III)

PTEN expression was studied both at mRNA and protein level. In the HER-2 positive cell lines, low PTEN (mRNA level equal to or below that obtained in a cell line with a genomic *PTEN* deletion) was observed only in two of the studied cell lines, the other of these being the JIMT-1. PTEN expression levels were found to be significantly lower in those cell lines with the *PIK3CA* mutation than in the wild-type cell lines ($p = 0.02$). Three out of five mutated cell lines showed PTEN mRNA levels below normal (equal to or above that obtained in a cell line with normal *PTEN* gene status), whereas in the *PIK3CA* wild-type cell lines, this number was only one out of four.

In the tumors studied, low PTEN mRNA expression was detected in 19.1% and negative PTEN protein expression detected by IHC in 9.9% of tumors. The concordance between IHC and qRT-PCR findings was poor (kappa coefficient 0.027, 95% confidence interval -0.327 to 0.380).

4.1 Clinicopathological characteristics of tumors with low PTEN expression (III)

Also PTEN protein expression was correlated with clinicopathological characteristics of the tumors. PTEN immunostaining was associated with ER negative tumor type ($p = 0.04$) while the PR status did not correlate with PTEN protein expression ($p = NS$). Basal-like (K5/14 positive) tumors were more often PTEN negative than tumors with positive PTEN immunostaining result although the association did not reach statistical significance ($p = 0.06$). A statistically significant association was detected between negative PTEN protein expression and receptor negative tumor type ($p = 0.003$). No correlation was detected between PTEN staining result and HER-2 status, patient age, distribution of axillary lymph node status, and Ki-67 expression ($p = NS$ for all).

This study showed that negative PTEN expression is associated with a different tumor type than *PIK3CA* mutations, although these two aberrations did co-exist to some extent. According to these results there is very low correlation between PTEN mRNA and protein expression.

DISCUSSION

1. Prevalence of HER-2 amplification in breast cancer (I)

HER-2 gene amplification was identified in breast cancer in 1985 (King et al. 1985). Two years later a group discovered HER-2 overexpression to be associated with a more aggressive type of breast cancer and thereby having prognostic importance (Slamon et al. 1987). The first drug for the treatment of HER-2 positive breast cancer (trastuzumab) was approved by FDA in 1998 and by European Commission in 2000. Treatment with trastuzumab is very expensive. The duration of therapy can be up to one year, which costs about 35 000 Euros in Finland. To be able to evaluate the expenses of the treatment as exactly as possible, it is important to know the number of patients.

Estimations about the proportion of HER-2 positive breast cancer of all breast cancers have varied between 10% and 40% (Cardoso et al. 2005). The earlier figures are based on immunohistochemical analyses, which are known to produce a certain amount of false positive results. Nowadays in situ hybridization is the predominant diagnostic method and it is shown to be more accurate. A commonly used phrase in publications is "20% to 30% of breast cancers show HER-2 oncogene amplification" but the prevalence of this breast cancer type has not been studied extensively. Therefore we wanted to find out the incidence and proportion of HER-2 positive breast cancer in the Pirkanmaa Hospital district, Finland. All tumors were analyzed by in situ hybridization method currently in use for determination of HER-2 status for clinical diagnosis. Thus the results of all study cohorts can be considered as comparable. The proportions of hormone receptor positive and negative tumors in each cohort equated all breast cancers diagnosed during respective time periods. This indicates that the cohorts were representative.

Our results showed that the proportion of HER-2 positive breast cancer had declined during the study period. In the meanwhile the incidence of all breast cancers did increase remarkably, which indicates that the incidence of HER-2 positive tumors had remained almost stable. According to our results the current proportion of HER-2 positive breast cancers of all breast cancers is less than 15%, which is lower than generally estimated. Also in a recent Swedish study nearly equal figures have been reported from years 2005-2007 (Rydén et al. 2009). Additionally the results of a publication studying the epidemiology of different breast cancer subtypes in two large cohorts of breast cancer patients in US showed the proportion of HER-2 positive tumors to be 15.3% (Kwan et al. 2009). In the earliest cohort of our study (1982-1986) 21.6% of all breast cancers were HER-2 amplified. This matches well with previously published findings from the

same hospital district (Kallioniemi et al. 1991), although that study was based on analysis of HER-2 protein overexpression by immunohistochemistry instead of detection of gene amplification.

There are few published studies covering the epidemiology of different molecular breast cancer subtypes and the results are somewhat contradictory. Published reports covering changes in the breast cancer incidence however show that the proportion of ER positive cancers and tumors in patients aged 45 years or older has increased during years 1980-2006 (Glass et al. 2007). We also found a significant increase in the proportion of estrogen receptor-positive tumors in this study (from 59.4% in 1982 to 1986 to 89.8% in 2004 to 2005). Positive estrogen receptor status is shown to be inversely associated with HER-2 amplification in breast cancer (Cardoso et al. 2005). HER-2 positive tumor type is also more common in patients of younger age (Anders et al. 2008). A change in the incidence of histological subtypes of breast cancer has been observed as well. The proportion of invasive lobular tumors has increased (Zhao et al. 2004, Cocquyt et al. 2005) and HER-2 amplification is known to be inversely correlated with the lobular tumor type (Hoff et al. 2002, Bane et al. 2005). All these findings about changes in the incidence of specific breast cancer types are in line with our results of decreased proportion of HER-2 positive breast cancer.

Although breast cancers are divided into subtypes according to pathological and molecular characteristics, the epidemiologic studies mostly have viewed breast cancer as a single disease with universal risk factors. There are only few articles studying the relationship between the known risk factors and the molecular subtypes. Most of the recognized risk factors influence the risk for breast cancer mainly through hormonal mechanisms and are therefore suggested to be more strongly related to the risk of luminal (ER positive) breast cancer. However, luminal tumors are classified into two groups according to HER-2 status, luminal A tumors being HER-2 negative and luminal B tumors, which are HER-2 positive. Therefore the hormonal risk factors probably do not affect both luminal subtypes equally. Particularly association of HRT use with breast cancer has been under intensive research recently. Exposure to exogenous estrogen is shown to be associated with hormone receptor positive breast cancer (Chen et al. 2004, Colditz et al. 2004). Kwan et al. (2009) have reported divergences in HRT use between patients with luminal A and luminal B tumors. These results propose that exposure to estrogen is not associated with HER-2 positive breast cancer. However, HER-2 overexpressing tumors are underrepresented in most studies and therefore no significant differences in HRT use of patients with HER-2 positive tumors compared with luminal breast cancer have been reported. Use of HRT has decreased in several countries during recent years. Epidemiological studies are needed in the future to find out whether this will affect the incidence of HER-2 positive breast cancer. HER-2 positive breast cancers are shown to constitute a group of heterogenous tumors and can be divided into various subtypes according to

gene expression. This, as well as some previous studies have also shown that HER-2 amplification and basal-like tumor type are not mutually exclusive (Laakso et al. 2005, Staaf et al. 2010).

A positive correlation has been shown between HER-2 amplification and older age at first birth or not breastfeeding although having children (Phipps et al. 2008, Kwan et al. 2009). Phipps et al. (2008) found the HER-2 overexpressing tumor type also to be associated with early age at menarche. However, this study combined the luminal tumors into one group despite the HER-2 status and therefore this result applies only for HER-2 positive and hormone receptor negative tumors. Usually high body mass index (BMI) is considered as a risk factor for breast cancer in postmenopausal women due to adipose tissue as a source of estrogen. However, HER-2 amplification has been suggested to be inversely associated with BMI independent of ER status of the tumor in postmenopausal women (Van Mieghem et al. 2007). Results of the different studies vary because the cohorts have included patients of different ages, menopausal statuses and ethnic groups and the numbers of triple negative and HER-2 overexpressing tumors have been small. Risk factors for HER-2 positive cancer remain to be unsolved and there is a need for further studies with large enough patient cohorts. Although HER-2 positive cancers account for only a relatively small part of all cancers, understanding of risk factors would be important due to the association with poor prognosis and due to special therapy of this tumor type (anti-HER2 drugs).

Although not being a risk factor, introduction of mammography screening has been also associated with increased incidence of breast cancer. In our data the diagnostic information was available for the latest cohort and we found that the HER-2 positive cancers were less often diagnosed by mammography screening than HER-2 negative tumors. The correlation between HER-2 status and diagnostic method of breast cancer has not been widely studied. A recent study shows underrepresentation of HER-2 positive and hormone receptor negative cases among the screen-detected tumors (Sihto et al. 2008). Similar results have been published also in earlier studies analyzing HER-2 protein expression in screen detected and interval cancers (Crosier et al. 1999, Porter et al. 1999). Studies comparing mammographic appearances show that the more aggressive HER-2 positive tumors are more often comprised of so called ill-defined masses, which are more difficult to detect as small than the spiculate lesions predominant in luminal tumors (Taneja et al. 2008). Screen detected cancers are more often ER and PR positive (Crosier et al. 1999, Porter et al. 1999) and thus HER-2 negative (Cardoso et al. 2005). Thus, bias caused by method of detection (screening/clinical) could be one explanation for the decreased proportion of HER-2 positive tumors of all breast cancers.

To our knowledge this was the first published study reporting the epidemiology of HER-2 positive breast cancer. Epidemiological studies covering the incidence of different types of

breast tumors are important for estimating the resources needed for cancer treatments. These studies also give more information about how the risk factors affect different types of breast cancer. Although the incidence of HER-2 positive breast cancer is rather low, the possible alterations in risk factors (increasing age of the primigravida, decrease in the use of HRT) might cause a change in the incidence of HER-2 positive breast cancer. Therefore also studies covering treatment of HER-2 positive breast cancer are needed in the future.

2. Resistance to HER-2 targeted agents (II)

Various kinds of targeted therapies are becoming more and more important in cancer treatment. The first targeted agent against HER-2 receptor was trastuzumab. Although it has been in clinical use for over 10 years, the mechanisms of action are not entirely clear. Trastuzumab offers significant clinical benefit to patients with HER-2 positive breast cancer. However, not all patients respond to therapy, even when trastuzumab is combined with chemotherapeutic regimens. Resistance can be either primary or acquired. Several different mechanisms are proposed to lay behind trastuzumab resistance; alterations in the PI3K-PTEN pathway, increased signaling from other receptors of the HER – family due to autocrine ligand expression, and low expression of HER-2 receptors. However, until now there is no clear understanding about the actual role of the different resistance mechanisms.

We used a model of intrinsic trastuzumab resistance in our study. The JIMT-1 cell line is derived from a patient with HER-2 positive breast cancer clinically resistant to trastuzumab (Tanner et al. 2004). Nearly all the *in vitro* studies covering trastuzumab resistance are conducted with only one or two cell lines. Therefore we chose to use a panel of HER-2 positive breast cancer cell lines previously reported to be trastzumab sensitive to find out the possible differences in the occurrence of resistance mechanisms. As a control we used a HER-2 negative breast cancer cell line. Also another HER-2 targeted agent, lapatinib, is accepted in clinical use and it is stated to have at least partially different mechanisms of action and resistance than trastuzumab. Thus we studied the effect of lapatinib on the same cell lines. The results showed that JIMT-1 as a super resistant cell line has several molecular resistance mechanisms, which probably are additive and result in a drug resistant cellular phenotype. JIMT-1 cells were also most resistant to both of the drugs studied.

HER-2 expression in cells was studied as the ability of the receptors to bind trastuzumab. To our knowledge, receptor expression in relation to the trastuzumab binding ability of the cells has not been previously studied on a panel of cell lines. JIMT-1 cell line showing the lowest receptor expression was also completely resistant to trastuzumab and the most sensitive cell

lines expressed remarkably higher levels of trastuzumab binding HER-2. However, the trastuzumab responses did not directly depend on the receptor expression in all the studied cell lines. This may be at least partly due to other resistance mechanisms in these cells. In previous *in vitro* studies the effect of trastuzumab is shown to be correlated with level of HER-2 expression (Merlin et al. 2002, Emlet et al. 2007). However, a recent study combining data from two-dimensional and three-dimensional assays with a large panel of cell lines did not either find association between trastuzumab sensitivity and HER-2 protein expression (O'Brien et al. 2010). Gene amplification is known to be correlated with HER-2 overexpression and also we detected an association between gene copy number and trastuzumab binding capacity of the cells. Although HER-2 expression is a well known indicator of trastuzumab efficacy, it clearly explains the drug response only partially. Even though cells would be expressing high levels of HER-2 receptors, binding of the antibody can be disturbed. The receptors can be truncated, which results in absence of trastuzumab binding site. Also MUC4 proteins are shown to mask HER-2, which prevents binding of trastuzumab (Nagy et al.2005). A similar role has also been proposed for hyaluronan (Pályi-Krekk et al. 2007). JIMT-1 cells are shown to express both of these proteins at high levels (Pályi-Krekk et al. 2007).

Crosstalk within the HER receptors is shown to be important for signal transmission. Other receptors of the family are activated following trastuzumab treatment as a compensation for suppression of HER-2 (Bender and Nahta 2008). Thereby the effective inhibition of cell growth by trastuzumab depends on the action of several receptors. One possible mechanism for increasing the effect of trastuzumab is to target several members of the receptor family simultaneously. Already in clinical use is lapatinib, which is a dual HER-1 and HER-2 inhibitor. It has also been shown that HER-3 would have the most important role in forming oncogenic units with HER-2 (Lee-Hoeflich et al. 2008). Preclinical trials are ongoing evaluating anti-HER receptor targeted drugs that block the entire receptor family as well as agents targeting different receptors selectively. Also combination of pertuzumab, which inhibits HER-2 dimerization, and trastuzumab has shown promising results in patients with HER-2 positive breast cancer.

Neuregulin 1 is a ligand of HER-3 and HER-4. Although it is known to have a role in cell transformation (Krane and Leder 1996) and metastatic spread (Atlas et al. 2003) and shown to be expressed in clinical breast tumors (Dunn et al. 2004), its role in HER-2 positive cancer has not been extensively studied. Ligand-binding is shown to be essential for dimerization and thereby activation of the receptor tyrosine kinases. We detected neuregulin 1 mRNA expression in two cell lines studied. JIMT-1 cells was other of these cell lines with 20 times greater expression than in UACC-812 cells. Previous *in vitro* studies have shown that presence of exogenous neuregulin 1 inhibits the effect of trastuzumab in HER-2 positive trastuzumab sensitive breast cancer cell lines

(Diermeier et al. 2005). Also the antiproliferative effect of the murine form of the antibody, 4D5, is reversed by neuregulin 1 on breast cancer cells (Motoyama et al. 2002). In addition to exogenous neuregulin 1 produced by the surrounding stroma, this growth factor can also exist as autocrine, meaning that the cells secrete it themselves. Xenograft tumors with acquired trastuzumab resistance have been reported to overexpress endogenous neuregulin 1 (Ritter et al. 2007). The gene encoding neuregulin 1 is located on 8p12, which is a known site for rearrangements in breast cancer (Adélaide et al. 2000, 2003). Breakpoints within the *NRG1* locus have been described in breast cancer cell lines (Adélaide et al. 2003) and tend to occur together with *FGFR1* amplification. This amplification has been reported in the JIMT-1 cell line (Rennstam et al. 2007).

Antibody-dependent cellular cytotoxicity is proposed to have an important role in the action of trastuzumab (Arnould et al. 2006, Musolino et al. 2008). We studied trastuzumab-induced ADCC reactions in the cell lines and our results showed only minor differences between the cells. Previously it has been shown that ability of trastuzumab to mediate ADCC is related to the level of HER-2 expression (Lewis et al. 1993). We did not detect any correlation between trastuzumab binding capacity and the ADCC efficacy in the HER-2 positive cells, although the variation in the level of trastuzumab binding receptors was wide. In HER-2 negative MCF-7 control cell line the ADCC reaction, however, was very low. Also the superresistant JIMT-1 cells expressed moderate ADCC activity; similar results have been obtained also in an earlier study using mouse xenografts. They reported also an effective ADCC based growth inhibition of JIMT-1 cells *in vitro* and *in vivo* (Barok et al. 2007). The contribution of ADCC activity in trastuzumab effect is remains unclear. Our results propose that the intrinsic resistance mechanisms may be unrelated to ADCC. This is not surprising, since ADCC is an indirect mechanism involving the immune system instead of cell signaling. According to current knowledge, the only mechanisms inhibiting ADCC would be those preventing binding of trastuzumab. Clinically there have been observed differences in the ADCC reactions between patients, but these are shown to be due to dysfunctional NK cells.

Lapatinib is proposed to have different mechanisms of action and thus also mechanisms of resistance than trastuzumab. It is currently in clinical use for HER-2 positive patients who have progressed on trastuzumab treatment. Nevertheless, median duration of lapatinib response is less than one year and majority of trastuzumab pre-treated patients do not respond to therapy (Geyer et al. 2006, Blackwell et al. 2009). Also our results showed cross-resistance between these two drugs. Mechanisms of lapatinib resistance are still under research.

According to the results of this study, it is likely that multiple mechanisms of resistance exist and that these mechanisms may vary among cell lines. Earlier studies covering trastuzumab resistance have mainly focused on a single resistance mechanism, and more

importantly, none have introduced a model of extreme drug resistance, such as the JIMT-1 cell line. In the present study, a panel of cell lines was studied in order to prevent a biased outcome, since the characteristics of different HER-2 positive cell lines may remarkably differ from each other. Clearly, the present results using nine HER-2 positive cell lines indicate that drug resistance is a complicated phenomenon resulting from different biological properties. Moreover, the results suggest that several co-existing mechanisms are required to evoke a resistant phenotype. Further studies are needed to clarify the significance of different resistance mechanisms and to detect measurable biomarkers for these mechanisms. An interesting question also is whether there is divergence in mechanisms contributing to acquired versus inherent resistance.

This study further emphasized the challenges in treatment of patients with HER-2 positive breast cancer. Response to treatment varies between patients and is impossible to predict. Although cell lines are widely used as *in vitro* models in cancer research, they have limitations and the results are not always totally applicable in the clinical setting. Our results show that incidence, significance and synergism of different resistance mechanisms should be studied also in clinical tumor samples. Also the patients with intrinsic and acquired resistance should be compared and probably considered as two different patient groups. Future studies should be aimed to finding of biomarkers as well as new strategies for treatment of patients with HER-2 positive breast cancer.

3. Altered PI3K-PTEN pathway in HER-2 positive breast cancer (II,III)

Activation of HER-2 and the other receptors of the HER family results in increased signaling in several pathways. One of the most important is the PI3K-PTEN signaling route. Deregulation of this cascade can be due to several divergent genetic aberrations, resulting in either decreased expression of PTEN, amplification or mutation of *PIK3CA* or amplification or mutation of AKT followed by uncontrolled signaling. HER-2 and other receptors of the HER family are important activators of this pathway. Functional PI3K-PTEN signaling is known to be critical for trastuzumab sensitivity. We studied two members of this pathway, oncogene *PIK3CA* and tumor suppressor *PTEN* in breast cancer cell lines and clinical breast tumor samples.

We detected *PIK3CA* mutations in five of the nine HER-2 positive cell lines and in one HER-2 negative cell line. Quantitative PCR analysis showed that PTEN mRNA expression was statistically significantly lower in cell lines with mutation than in wild type cell lines. Previously it has been shown that these two aberrations would be mutually exclusive (Saal et al. 2005), although the more recent studies have presented results that are in line with ours (Pérez-Tenorio et al. 2007, Stemke-Hale et al. 2008). Both of these defects were detected in the JIMT-1 and MDA-MB-361

cell lines, which showed no or only minor responsiveness to trastuzumab and lapatinib. Both *PIK3CA* mutations and low PTEN expression are shown to decrease trastuzumab activity in breast cancer (Nagata et al. 2004, Berns et al. 2007). Several studies propose that PTEN deficiency would not have a role in lapatinib resistance (Xia et al. 2007, Johnston et al. 2008, O'Brien et al. 2010). Our study however, indicates a correlation between low PTEN as well as mutated *PIK3CA* and *in vitro* sensitivity to lapatinib, which has been reported also by Eichhorn et al. (2008). Their study showed association of two *PIK3CA* hot spot mutations and lapatinib resistance. In our study the most trastuzumab-resistant cell line JIMT-1 had a more rare type of *PIK3CA* mutation. However, this mutation has been shown to act as a strong transformer, as well as to constitutively activate the downstream signaling cascade (Gymnopoulos et al. 2007). This cell line also has a very low level of PTEN expression and other properties that explain its high resistance.

In HER-2 positive clinical tumors the mutation rate of *PIK3CA* was 14.5%, which is considerably lower than in cell lines. Association of *PIK3CA* mutations with HER-2 negative cancer type has been shown also in other studies (Perez-Tenorio et al. 2007, Kalinsky et al. 2009) although opposing results have also been presented (Saal et al. 2005). Our study consisted solely of patients not treated with trastuzumab and the patients with HER-2 positive and *PIK3CA* mutation positive tumors had a shorter breast cancer specific survival when compared with the wild type cancers. Although the mutation positive and HER-2 positive tumor type was uncommon, it showed to be very aggressive. Since mutated *PIK3CA* has been associated with resistance to HER-2 targeted agents, it would be important to study the breast cancer specific survival in trastuzumab and also lapatinib treated patients. Positive HER-2 status is generally correlated with poorer survival. Nevertheless recent gene expression studies have identified groups of HER-2 positive tumors with different biologic characters and outcomes (Staaf et al. 2010). Our results suggest that mutated *PIK3CA* could be one important feature associated with poor prognosis in HER-2 positive cancer.

4. Characteristics of tumors with aberrant PI3K-PTEN signaling pathway (III)

Our results confirmed the opinion that *PIK3CA* mutations and low PTEN expression are common in breast cancer. The *PIK3CA* mutation rate in the clinical breast tumor samples analyzed was 29.5%, which is in line with the previously published results of sequencing studies (Saal et al. 2005, Wu et al. 2005). Most of the mutations were located in the known hot spots in the two exons encoding the helical and kinase domains of the protein. Exon 20 mutations were predominant, which is typical for breast cancer (Saal et al. 2005). The mutations located outside the hotspot areas are rarer but

have previously been reported to occur in breast cancers (Campbell et al. 2004, Perez-Tenorio et al. 2007). Also these mutations are shown to have oncogenic potential (Gymnopoulos et al. 2007). All detected mutations were located in different tumors, although in some studies existence of double mutations in breast cancers has been reported (Lee et al. 2005, Saal et al. 2005). We detected one mutation in exon 9, which, according to published data is not previously shown to occur in breast cancer.

Low PTEN mRNA expression was detected in 19.1% of the samples and loss of PTEN protein in 9.9% of the samples studied. The proportion of PTEN protein negative breast cancers has been reported to vary between 8-48% (Fujita et al. 2006, Saal et al. 2005, Nagata et al. 2004). Our results also suggest that PTEN as analyzed by qRT-PCR and IHC are not comparable. A small proportion of the samples showing normal mRNA level by qRT-PCR was found to be negative by immunostaining. Additionally, most tumors with low level of PTEN mRNA expressed positive protein staining. This finding is in contrast with Marty et al. (2008), who analyzed a small amount of selected breast tumor samples and detected a statistically significant correlation between PTEN protein and mRNA expressions by microarray. Regulation of *PTEN* expression is known to be complex. It has been shown that the accumulation of PTEN protein may result from increased protein stability instead of regulation of its transcription (Li et al. 2007). This may be one explanation for our finding of tumors with low mRNA expression but high protein expression. The results indicate that the mRNA expression measured with quantitative PCR analysis would not reflect the level of PTEN protein expression.

We correlated the *PIK3CA* mutation status and PTEN protein expression with clinicopathological characteristics of the tumor samples studied. Although these two aberrations did co-exist to some extent, we could still recognize two different tumor types related to them. *PIK3CA* mutations were associated with hormone receptor positive, HER-2 negative breast cancers while low PTEN expression was more common in the triple negative and basal-like tumors. There are some studies proposing that aberrations in both of these members of the PI3K-PTEN signaling pathway would be related with common tumor characteristics (Perez-Tenorio et al. 2007, Stemke-Hale et al. 2008). Yet most of the studies are in line with our results showing that *PIK3CA* mutations correlate with the more favourable luminal disease type (Kalinsky et al. 2009, Michelucci et al. 2009) while PTEN defects are associated with hormone receptor negative and HER-2 negative tumors (Marty et al. 2008). In all, the relationship between mutated *PIK3CA* and other tumor characteristics is still unclear and not all studies have been able to find significant correlations between mutations and clinicopathological features in breast cancer (Loi et al. 2010). One explanation for this could be that some of the studies are carried out with relatively small amounts

of tumors or the tumors included in the study have been selected by some characteristics. The strength of our study is a relatively large population of primary invasive breast tumors.

Activating *PIK3CA* mutations are reported to have several gain of function mechanisms. Mutations result in lipid kinase activity independent of the regulatory subunit of the protein, change interactions with other proteins or cell membrane or enhance the stability of the protein (Gymnopoulos et al. 2007). We studied by immunohistochemical analysis whether the mutations would result in protein overexpression or accumulation. In breast cancer cell lines the mutation status appears to correlate with protein expression (www.protainatlas.org). Also in one previous study with clinical breast tumor samples the *PIK3CA* mutations have been associated with protein overexpression (Lerma et al. 2008). However, we did not find any correlation between mutation status and protein expression in the breast cancers analyzed. At the moment there are also two published studies that have correlated PIK3CA protein expression with breast cancer characteristics and patient outcome. The results of these studies are somewhat contradictory with each other (Aleskandarany et al. 2010, Carvalho et al. 2010) as well as with our results from the mutation analyses. One possible explanation for the discrepancy between different publications could be variations in the sensitivities of the antibodies used. However, we used the same antibody than Aleskandarany et al., thereby this possibility should be excluded. Our stainigs were analyzed with an image analysis software, which should improve the reliability of the results. Nevertheless, in our study the PIK3CA IHC results did not correlate with any of the clinicopathological characteristics of tumors or patient survival. These discrepancies in the results of different studies indicate that IHC staining probably is not a reliable tool for identifying patients with *PIK3CA* mutations.

5. Association of mutated *PIK3CA* with breast cancer specific survival (III)

We detected a trend towards late mortality in tumors with mutated *PIK3CA*. The divergence of the survival curves was observed after ten years of follow-up. Interestingly, a similar survival curve was observed also for ER positive tumors with *PIK3CA* mutation when compared with ER positive wild type tumors. Some studies have not found any correlation between patient outcome and *PIK3CA* mutation status (Saal et al. 2005, Michelucci et al. 2009). This may be due to relatively short follow-up times in many analyses. Nevertheless, also poor survival during the first years of follow-up has been reported to be associated with *PIK3CA* mutations (Li et al. 2006, López-Knowles et al. 2010). Surprisingly, mutated *PIK3CA* has also been associated with better survival when compared with wild type tumors (Kalinsky et al. 2009) and also in ER positive tumors with a

follow-up up to 14 years (Loi et al. 2010). Differences in survival times have also been associated with location of the mutation (Barbareschi et al. 2007, Lai et al. 2008). These conflicting results indicate that although having strong tumorigenic effects, PIK3CA alone would not be a sufficient prognostic factor. Also differences in the treatments patients have received affect the clinical consequences and make the comparison of different studies complicated.

Until now, even the few long-term follow-up studies have not revealed any other prognostic indicators for late mortality during long follow-up time than positive ER status; usually the effect of unfavorable clinicopathological factors are seen as increased mortality from the beginning of the follow-up. There is a need to identify markers related to late breast cancer mortality, the mechanisms of which are poorly understood. Our results may implicate that patients with ER positive and *PIK3CA* mutated breast cancer are in higher risk of late breast cancer mortality and are in need of longer hormonal adjuvant treatment. In our study material most of the patients had not received any kind of adjuvant therapy. Therefore this hypothesis should be further studied in patients who have been treated with more modern and effective adjuvant treatment.

SUMMARY AND CONCLUSIONS

The main conclusions from this study are:

1. HER-2 positive breast cancer is not as common as assumed. Proportion of HER-2 amplified tumors has decreased during years 1982-2005 indicating that the incidence of HER-2 positive breast cancer has remained stable. Meanwhile the overall incidence of breast cancer has increased.

2. The known risk factors of breast cancer e.g. use of menopausal hormone replacement therapy may not affect HER-2 positive breast cancer at the same magnitude as HER-2 negative cancer.

3. Behind trastuzumab resistance may be concomitant mechanisms, which probably act in concert to produce a drug resistant cellular phenotype and the molecular mechanisms underlying drug resistance may be additive.

4. Mutated *PIK3CA* and low PTEN expression are common in breast cancer and they are characteristic for different breast cancer subtypes. PIK3CA protein expression appears not to be suitable as a surrogate marker for *PIK3CA* mutations.

5. Mutated *PIK3CA* could be a marker for late mortality in breast cancer, also in the group of ER positive tumors. Co-existence of *PIK3CA* mutations and HER-2 amplification are rare but this tumor type showed to be very aggressive.

AKNOWLEDGEMENTS

This study was performed in the Cancer Biology Group, Institute of Medical Technology, the University of Tampere, during the years 2006-2010. Professor Olli Silvennoinen, M.D., Ph.D., the director of the Institute of Medical Technology is acknowledged for providing the research facilities for this study. I also thank the Tampere Graduate Program in Biomedicine and Biotechnology for providing me with the graduate school position to carry out this work.

I am grateful to my supervisor Professor Jorma Isola for the opportunity to perform my doctoral studies in his research group. I thank him also for sharing his impressive knowledge on breast cancer and introducing me to the world of science. Professor Tapio Visakorpi, M.D., Ph.D. and Docent Ritva Karhu, Ph.D. are thanked for their support as thesis committee members. The official reviewers of this thesis, Professor Johanna Ivaska, Ph.D. and Docent Sirpa Leppä, M.D., Ph.D. are aknowledged for their valuable comments. I also owe my gratitude to my co-authors, Anssi Auvinen, M.D., Ph.D., Docent Minna Tanner, M.D., Ph.D., Professor Mark Barok, M.D., Ph.D., Synnöve Staff, M.D., Ph.D., Jukka Pitkänen, M.D., Ph.D., Päivikkki Hemmilä, Ph.D., Joanna Ilvesaro, Ph.D., and Vilppu Tuominen, M.Sc. for their invaluable contribution to this work.

I am grateful to all past and present members of the Cancer Biology Group for their help during this work, especially Jukka Pitkänen, M.D., Ph.D. for his guidance in the beginning of my research project and Vilppu Tuominen, M.Sc. for always having time and patience to help me with my computer crises. Ms. Sari Toivola and Ms. Kristiina Salonoja are acknowledged for their skillful technical assistance. Warm thanks go to my unofficial thesis committee; you were always there for me through thick and thin. Thank you for the fun we have had both at work and outside the lab, even in the middle of the week. You are not just workmates, you are friends! I am also grateful to my fellow PhD students Marjut Salo, Lic. Sc. (Econ.) and Tiina Riihimäki, M.Sc. for peer support and amusement. The encouragement and sparkling wine we have shared have been so important to me.

I thank my dear friends, all the new ones I have made here in Tampere and those who have remained as part of my life although living elsewhere. I am grateful for the support, patience and entertainment you have provided. I also want to mention late Adi and the newcomer Otso for being loyal companions and for helping me to clear my mind during long walks and trainings.

Finally, I am grateful to my family, as well as to my other relatives, especially aunts and cousins with their lovely children, for all the support I have received. I know I can always count on you.

Tampere, October 2010

Katri Köninki

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