Linköping University medical dissertations, No. 1607

Long-term prognostic and predictive factors in hormone receptor positive breast cancer

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Linköping 2018

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Printed in Sweden by LiU-Tryck, Linköping, Sweden, 2018

ISBN: 978-91-7685-372-6

ISSN: 0345-0082

Till Linnéa

*Jag genar bland blomstrande snår
och här finns så mycket gott man vill smaka.
Men när de plöjer små färdiga spår,
ja, då får jag lite lust att springa över alltihop och tillbaka*

- Lars Winnerbäck

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Abstract

The breast cancer survival in Sweden is good (almost 90 % 5-year relative survival) and has increased over time. For women with hormone receptor negative tumors, most relapses occur within the first 5 years after diagnosis. Thereafter the recurrence risk decreases rapidly. For women with estrogen receptor positive (ER+) tumors the annual risk for late recurrences is 1 – 2 %, even after 5 years of endocrine therapy. This risk accumulates so that approximately 25 % of the patients that are recurrence-free after five years from diagnosis may experience a relapse within further 15 years of follow-up. The relatively high long-term risk calls for identification of prognostic and predictive markers with long-term effect. Though, the number of such markers with proven significance is limited. Of the clinical characteristics, only nodal status and to some extent tumor size and tumor grade have been shown to have long-term prognostic value. In this thesis, we propose long-term prognostic and predictive markers for breast cancer.

In **paper I**, we suggest the protein v-akt murine thymoma viral oncogene homologue 2 (AKT2) as a long-term prognostic marker among patients with ER+ tumors. In our study, besides nodal status, AKT2 was the only factor with long-term prognostic value. This is in accordance with some other studies, though we also showed that the significance of AKT2 was limited to ER+ tumors and that the impact increased with higher ER expression.

Approximately 75 – 85 % of the ER+ tumors are also progesterone receptor positive (PR+). ER+/progesterone receptor negative (PR-) tumors are considered to be more aggressive and patients with such tumors are often treated with chemotherapy. In this group, more specific subgroups for targeted therapy are needed.

Whereas ER has long been established as a predictive factor regarding tamoxifen benefit, the role of PR has not been clarified to date. In **paper II**, we showed that PR status adds predictive value to ER considering the long-term benefit from tamoxifen.

In **paper III**, we aimed to identify new prognostic markers among patients with ER+ tumors. Systemically untreated patients with ER+/PR- tumors and high expression of the Ras-related protein RAB6C (RAB6C) had reduced distant recurrence rate. Therefore, we suggest RAB6C as a candidate marker for subgroup division among patients with ER+/PR- tumors.

According to the results from paper II, there might be subgroups of patients with ER+/PR- tumors that do benefit from tamoxifen. The aim of **paper IV** was to identify such subgroups. Here, we suggest that patients with ER+/PR- tumors and low RAB6C expression do benefit from tamoxifen.

The results from this thesis may encourage further studies for more specific subgroup divisions. Such studies may lead to changes in the management program, where some patients with ER+ tumors should receive prolonged or more intense treatment and others reduced treatment based on the pathological markers AKT2, PR and RAB6C.

List of papers

This thesis is based on the following original papers, which are referred to in the text by their Roman numerals.

- I. **Helena Fohlin**, Gizeh Pérez-Tenorio, Tommy Fornander, Lambert Skoog, Bo Nordenskjöld, John Carstensen, Olle Stål. *Akt2 expression is associated with good long-term prognosis in estrogen receptor positive breast cancer*. Eur J Cancer. 2013 Apr;49(6):1196-204.
- II. Anna Nordenskjöld*, **Helena Fohlin***, Tommy Fornander, Britta Löfdahl, Lambert Skoog, Olle Stål. *Progesterone receptor positivity is a predictor of long-term benefit from adjuvant tamoxifen treatment of estrogen receptor positive breast cancer*. Breast Cancer Res Treat. 2016 Nov;160(2):313-322.
- III. **Helena Fohlin**, Tove Bekkhus, Josefine Bostner, Tommy Fornander, Bo Nordenskjöld, John Carstensen, Olle Stål. *RAB6C is an independent prognostic factor in estrogen receptor-positive/progesterone receptor-negative breast cancer*. Submitted.
- IV. **Helena Fohlin**, Tove Bekkhus, Josefine Bostner, Tommy Fornander, Bo Nordenskjöld, John Carstensen, Olle Stål. *Low RAB6C expression predicts tamoxifen benefit in estrogen receptor-positive/progesterone receptor-negative breast cancer*. Manuscript.

*The authors contributed equally

Populärvetenskaplig sammanfattning

Biologiska markörer påverkar långtidsöverlevnad för bröstcancerpatienter

Bröstcancer är en vanlig sjukdom som drabbar över 8 000 kvinnor varje år i Sverige.

Samtidigt som allt fler kvinnor insjuknar har överlevnaden förbättrats.

Femårsöverlevnaden är nästan 90 % och tioårsöverlevnaden nära 80 %. Många patienter lever alltså länge efter diagnos, men återfallen kan komma sent. Hur stor risk en patient har för återfall beror delvis på tumörens egenskaper och påverkan kan vara olika stark på kort respektive lång sikt.

Två av de viktigaste tumöregenskaperna är hormonreceptorerna för östrogen och progesteron. Om tumören är östrogenreceptorpositiv (ER+) eller progesteronreceptorpositiv (PR+) betyder det att cancercellerna har receptorer för östrogen respektive progesteron. Receptorerna kan ta upp signaler från dessa hormoner och stimulera cellerna att växa. För kvinnor med tumörer som saknar dessa receptorer inträffar de flesta återfallen inom fem år efter diagnos. Därefter sjunker återfallsrisken kraftigt. Men de flesta tumörerna är ER+ och i dessa fall har patienterna nytta av antihormonell behandling. Denna behandling kan antingen sänka hormonhalterna eller blockera hormonreceptorerna så att cancercellerna får svårare att överleva. På kort sikt är prognosen god, men trots behandling kommer i snitt var fjärde patient som efter fem år från diagnos lever utan tecken på återfall att få det inom ytterligare 15 år.

Trots den relativt höga långtidsrisken är det ont om markörer med bevisad effekt för att kunna särskilja patientgrupper med olika god prognos på lång sikt. Sådana långtidsmarkörer skulle kunna bidra till att identifiera patienter som har nytta av förlängd behandling respektive patienter som kan slippa onödig behandling. Några tidigare studier har indikerat att patienter har bra prognos på lång sikt om det i

cancercellerna finns mycket av ett protein som heter AKT2. Vår studie har visat samma resultat, men vi har också sett att det är begränsat till ER+ tumörer och att betydelsen av AKT2 ökade ju fler östrogenreceptorer tumören har.

Cirka tre av fyra ER+ tumörer är också PR+. Våra resultat har visat att patienter som har tumörer som är både ER+ och PR+ på lång sikt har större nytta av antihormonell behandling jämfört med tumörer som har östrogenreceptorer, men saknar progesteronreceptorer (ER+/PR-). Den senare tumörgruppen anses mer aggressiv och patienterna ges ofta tilläggsbehandling i form av cellgifter, som kan ge svåra biverkningar som följd. I denna patientgrupp är det betydelsefullt att identifiera fler undergrupper för att kunna ge mer målriktad och skonsammare behandling. I våra studier har vi sett att patienter med ER+/PR- tumörer och som varken har fått cellgifter eller antihormonell behandling har bättre prognos om cellerna innehåller mycket av proteinet RAB6C. När vi vidare undersökte om det finns undergrupper bland patienter med ER+/PR- tumörer som har större nytta av antihormonell behandling, gällde detta för tumörer som innehåller mindre mängd RAB6C.

Även om det behövs ytterligare studier för att bekräfta resultaten, kan de i framtiden ha betydelse inom praktisk sjukvård. Patienter som har god överlevnadchans även med minskad behandling bör informeras om nyttan respektive riskerna med behandling, medan andra möjligtvis ska rekommenderas mer intensiv eller förlängd behandling.

Abbreviations

AKT	v-akt murine thymoma viral oncogene homologue
AP-1	activator protein 1
BCS	breast conserving surgery
BRCA	breast cancer gene
CISH	chromogenic in situ hybridization
CMF	cyclophosphamide, methotrexate and fluorouracil
EBCTCG	Early breast cancer trialists' collaborative group
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EIA	enzyme immunoassay
ER	estrogen receptor
ESR	estrogen receptor gene
FEC	fluorouracil, epirubicin, cyclophosphamide
FFPE	formalin-fixed, paraffin-embedded
FGFR	fibroblast growth factor receptor
FISH	fluorescence in situ hybridization
G1 phase	first gap phase
GDP	guanosine diphosphate
GTP	guanosine triphosphate
GPCR	G-protein-coupled receptors
HER2	human epidermal growth factor receptor 2
HOXB13	homeobox B13
IF	isoelectric focusing
IGF1R	insulin like growth factor 1 receptor
IL17BR	interleukin 17 receptor B
HR	hazard ratio
IGF	insulin-like growth factor
IHC	immunohistochemical staining
IQR	interquartile range
Ki-67	marker of proliferation Ki-67
M	presence of distant metastasis
M phase	mitotic phase
MAP	mitogen-activated protein
mTOR	mammalian target of rapamycin
mTORC2	mammalian target of rapamycin complex 2
N	degree of spread to the regional lymph nodes
NCBI	National center for biotechnology information
NF- κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
NHG	Nottingham histological grade

p53	tumor protein p53
pAKT	phosphorylated AKT
PDK-1	phosphoinositide dependent kinase 1
PI3K	phosphatidylinositol-3-kinase
PIK3CA	phosphatidylinositol 3-kinase catalytic subunit alpha
PIP ₃	3'-phosphorylated phosphoinositide
PR	progesterone receptor
PTEN	phosphatase and tensin homolog
RAB6C	RAB6C, member RAS oncogene family
RPS6K1	ribosomal protein S6 kinase 1
RT	radiotherapy
RTK	receptor tyrosine kinase
S phase	synthetic phase
SISH	silver enhanced in situ hybridization
SNP	single-nucleotide polymorphism
SP-1	specificity protein 1
STAT3	signal transducer and activator of transcription 3
STEPP	subpopulation treatment effect pattern plot
T	size of the primary tumor
TAM	tamoxifen

Background

Epidemiology and risk factors

Breast cancer is the most common cancer among women worldwide [1]. In Sweden, the risk for a woman to develop the disease before the age of 75 is 11 % [2]. It represents 30 % of all female cancer, but only 13 % of the cancer deaths, reflecting the good prognosis [3]. The median age for being diagnosed with breast cancer is 65 and 17 % are younger than 50 years old (Swedish Cancer Registry). Specific genetic mutations are strong risk factors for developing breast cancer, often at younger age compared to sporadic cancer [4]. Such germline mutations account for 5 – 10 % of all breast cancers, where the most well-known are *BRCA1* and *BRCA2* [5]. Another strong risk factor is detection of high density breast tissue [6]. Many other established risk factors are hormonally related. Early menarche, late menopause and short menstrual cycles increase the risk, as well as use of exogenous hormones (oral contraceptive pill and combined postmenopausal hormone replacement therapy) , whereas younger age at giving birth to the first child, going through many full-time pregnancies and breastfeeding are protecting factors [7-12]. In some cases, the risk factors differ depending on age. Obesity implies higher risk among postmenopausal, but is a protecting factor among premenopausal women [13]. Other potentially preventable factors include radiation exposure, high alcohol consumption and lack of physical activity [14].

Even adjusting for the fact that the population is getting older, the incidence of breast cancer has increased during the last decades (Fig. 1). One reason for that is probably that women are getting pregnant later in life and having fewer children [15]. Another contributing factor may be that women in certain periods were offered hormone replacement therapy due to menopause symptoms to a larger extent [10-

12, 14, 16, 17]. In general, an increase in a specific disease does not always reflect true incidence increase, but can be due to improved detecting methods. In the late 80's the breast cancer incidence in Sweden increased as mammography screening was introduced [18]. This leads to earlier detection and improved survival [19, 20]. Another way to analyze survival differences over time is to observe mortality rates according to the Swedish cause of death register. Also from that point of view, the breast cancer mortality has decreased (Fig. 1).

*using the 2000 Sweden standard population

Fig. 1 Breast cancer incidence and mortality in Sweden, based on data from the National Board of Health and Welfare (Socialstyrelsen) [3].

Prognostic and predictive factors

Prognostic factors describe the clinical outcome for the patients without treatment, in contrast to predictive factors which reflect the chance that the patients will respond to a specific treatment. The most important prognostic factors are the stage of the tumor (TNM), Nottingham histological grade (NHG), Ki-67 and age. Predictive factors include the estrogen receptor (ER), the progesterone receptor (PR) and the human epidermal growth factor receptor 2 (HER2). Some biomarkers are considered to be both prognostic and predictive, for example ER and HER2 [21].

Stage (TNM)

The stage is determined by the tumor size (T), lymph nodal involvement (N) and presence/absence of distant metastasis (M), and is the most significant prognostic factor. Stage I include small tumors with no lymph nodal involvement (N0), whereas stage II and stage III include larger tumors and/or lymph nodal involvement. In presence of distant metastasis (M1), the disease is classified as stage IV, independently of T and N. Most patients are diagnosed with stage I or stage II disease and have good survival.

Nottingham histologic grade (NHG)

NHG represents the aggressive potential of the tumor and is determined by three different components: tubular differentiation (how well the tumor cells try to recreate normal glands), pleomorphism (how ugly the tumor cells look) and mitosis (how much the tumor cells are dividing). Each of these features is scored and added to a final score which is used to assess the tumor grade 1, 2 or 3 representing low, intermediate or high.

Ki-67

The nuclear protein Ki-67 is a proliferation marker which is determined by how fast the cells are dividing. The more tumor cells that are positive for Ki-67, the more rapidly the tumor tissue is forming new cells. Ki-67 is associated with tumor grade (NHG) and high Ki-67 confers poorer prognosis [22-24].

Age

Younger women (<35 years old) at the time of diagnosis have higher risk of dying from the disease compared with middle-aged counterparts, partly because young age is associated with later stage and more aggressive tumor types [25, 26]. Women older than 70 years old also have lower 5-year relative survival compared with middle-aged patients. Here, a contributing factor may be that elder women are receiving less aggressive treatment [27].

The estrogen receptor (ER) and the progesterone receptor (PR)

Approximately 80 % of the breast cancer tumors are estrogen receptor positive (ER+) and out of these are 75 - 85 % also progesterone receptor positive (PR+). In the 1980's ER and PR were suggested as predictive factors for endocrine treatment [28-30]. Endocrine treatment includes tamoxifen, which acts in a way that blocks the hormone receptors and aromatase inhibitors which lower the hormone levels. Hormone receptor positivity is associated with good 5-year relative survival. However, despite endocrine treatment, the annual long-term risk for breast cancer recurrence is higher than for patients with hormone receptor negative tumors (Fig. 2).

Fig. 2 Annual recurrence rate per 100 person years at risk divided by ER status. The figure is based on data from Paper II - IV.

The human epithelial receptor 2 gene (HER2)

HER2 is amplified in approximately 15 % of the breast cancer cases and associated with more aggressive clinical behavior. The gene encodes a transmembrane tyrosine kinase growth factor receptor which acts through a variety of pathways, including the mitogen-activated protein (MAP) kinase and PI3K/AKT pathways. Enhanced activity in these pathways eventuate proliferation, angiogenesis, altered cell-cell interaction, increased cell motility, metastases and resistance to apoptosis [31]. In the 2000's, HER2 was identified as a target gene for response to trastuzumab, an antibody binding to the receptor [32-34]. This therapy significantly improved the disease-free survival for patients with HER2 amplified breast cancer [32, 35, 36].

Subtypes

Breast cancer may be divided into different treatment groups based on the routinely evaluated pathological markers described above. More recently, molecular profiling methods have been used to define clinical relevant molecular subtypes: luminal A-like, luminal B-like, HER2 subtype, and basal-like [37-39]. These subtypes can essentially be categorized into the pathological subtypes using ER, PR, Ki-67 and HER2 [40].

Survival

The breast cancer survival in Sweden is good (almost 90 % 5-year relative survival) and has increased over time [26, 41, 42]. For women with hormone receptor negative tumors, most relapses occur within the first 5 years after diagnosis. Thereafter the recurrence risk decreases rapidly. For women with hormone receptor positive tumors the cumulative 15-year risk for recurrences is 30 %, even after 5 years of tamoxifen [43, 44]. A recent meta-analysis including patients with ER+ tumors showed that the risk of distant recurrence after five years of endocrine therapy with 20-years of follow-up was up to 41 % [45]. This shows the importance of identifying prognostic and predictive markers with long-term effect on survival. At present, the number of such markers with proven long-term significance is limited. Among the clinical characteristics, only nodal status and to some extent tumor size and tumor grade have been shown to have long-term prognostic value [45, 46]. Table 1 shows the relative survival for breast cancer patients in two Swedish health care regions divided by stage at diagnosis.

Table 1 Stage distribution and age-standardized relative survival for patients diagnosed with breast cancer between 2004 and 2008 in the Western and South-East health care regions in Sweden.

Stage		Breast cancer patients (%)	5-year relative survival (%)	10-year relative survival (%)
I	T < 20 mm, N0, M0	45	97	91
II	T 20 - 50 mm, N 0 – 3, M0 <i>or</i> T < 20 mm, N ≥ 1, M0 <i>or</i> T > 50 mm, N0, M0	40	87	77
III	T 20 - 50 mm, N ≥ 4, M0 <i>or</i> T > 50 mm, N 1 – 3, M0	12	65	46
IV	Any T, any N, M1	3	20	11
Total		100	86	76

Surgery

Breast surgery

Nowadays, most patients are operated with breast-conserving surgery (BCS). The proportion of BCS compared with mastectomy has increased due to smaller tumor size, partly because of mammography screening. For unifocal tumors, it has been shown that the survival rate for BCS combined with radiotherapy against the breast is similar with that of mastectomy [47-49].

Axillary surgery

The main purpose of axillary surgery is tumor staging, in order to provide guidance for further adjuvant treatment. Node status is mostly determined by sentinel-node biopsy. If the sentinel node is positive, axillary dissection is performed.

Earlier, axillary dissection was the standard technique, but the introduction of sentinel-node biopsy has changed the approach as the status of the axilla can be established with less morbidity for patients when compared with complete axillary dissection.

Adjuvant treatment according to the Swedish national management program

Endocrine therapy

Generally, patients with hormone receptor positive breast cancer receive endocrine therapy. If the tumor is small (≤ 10 mm), lymph node negative and luminal A (strong ER+ and PR+, low proliferation and HER2-) abstaining of endocrine therapy may be

considered. Premenopausal women with intermediate or high risk are recommended tamoxifen for at least five years. For postmenopausal women the primary choice of endocrine treatment is aromatase inhibitors.

Chemotherapy

Chemotherapy is standard treatment for patients who have axillary node positive breast cancer as well as axillary node negative with other risk factors. Patients with hormone receptor negative breast cancer normally also receive chemotherapy. It is mostly given in a combined treatment of FEC (FEC comes from the initials of the drugs used: fluorouracil, epirubicin, cyclophosphamide) followed by taxane based chemotherapy.

Radiotherapy

Radiotherapy (RT) against the breast is standard therapy for patients operated with breast conserving surgery. For radical mastectomized patients no RT against the breast is given unless the tumor is larger than 50 mm.

RT against the axilla is not recommended if there are no lymph node metastases. Though, it is standard treatment if there is at least one macro metastasis. If the tumor is of grade 1 and there is only one macro metastasis, no RT may be considered. If only one micro metastasis is found, RT against the axilla or chest wall is normally not provided unless the tumor is larger than 50 mm.

Targeted therapy

Treatment for one year with trastuzumab together with chemotherapy (taxane) is recommended for patients with HER2+ breast cancer. The HER2 positivity should be

confirmed by strong immunohistochemical expression (IHC 3+) or FISH/CISH/SISH positivity.

Side effects from treatment

Endocrine therapy may worsen symptoms from lower levels of estrogen and may cause hot flashes, sweats, dry mucous membranes and musculoskeletal pain [50].

Side-effects from chemotherapy include fatigue, nausea and vomiting, hair loss and also potentially life-threatening effects such as neutropenia [51].

Radiotherapy is associated with radiation fibrosis and damage to the skin [52] and targeted therapy is mainly associated with dermatological side-effects. [53]

Prognostic gene expression profiles

Several studies are concerned with evaluating the patient's individual risk of recurrence by combining expression profiles of cancer-related genes. One of the first genomic assays developed for this purpose was MammaPrint, which combines the gene expression of 70 genes [54]. It requires freshly frozen tissue collected into an RNA preservative solution, which makes it more complex to conduct than other panels [55]. Although MammaPrint has been shown to have prognostic significance, it performs best as a predictor of distant metastasis during the first 5 years after treatment.

PAM50 is another genomic assay generated from the expression of 50 genes. It has recently been included in recommendations for decisions on adjuvant systemic

treatment for patients with negative lymph nodal status, hormone receptor positive and HER2 negative disease [56-58].

With Oncotype DX, the expression of five reference genes and 16 cancer-related genes is used [59]. A mathematical computation of the weighted expression of each gene results in a recurrence score which may be divided into three categories with low, intermediate and high risk [55]. BCI is another algorithmic gene expression-based signature which also divides the patients into three groups, but involving fewer genes [60]. The algorithm is based on the gene expression ratio of HOXB13 and IL17BR combined with the additive expression values of five cell-cycle genes. It has been shown to provide significant prognostic value for patients with ER+ and lymph node negative disease.

PAM50, Oncotype DX and BCI are all performed with RNA from formalin-fixed, paraffin-embedded (FFPE) samples.

There are also other genomic assays which have been shown to have long-term predictive significance for early-stage breast cancer, but none of them was specifically developed for predicting late recurrence [46, 60-62].

Several genomic predictors are already, or will be, part of routine clinical practice in the future [55, 63]. Even though they have been shown to add predictive significance for early-stage breast cancer, there are some limitations. The cut-off for gene panels varies between studies, making it difficult to identify correct cut-off points for clinical utility. Other issues associated with some gene panels are technical complexity or how to deal with patients identified with intermediate risk. The accuracy in predicting late recurrence in ER+ breast cancer is another issue to be solved.

The cell cycle

The purpose of the cell cycle is to accurately duplicate the DNA in the cell and physically split into two daughter cells. This is done in two major steps, the interphase and the mitotic (M) phase. The interphase involves three steps, the first gap (G1) phase, the synthetic (S) phase and the second gap (G2) phase. During the G1 phase, the cell grows physically larger, copies organelles, and makes molecular building blocks that are needed in later steps. During the S phase, the cell synthesizes a complete copy of the DNA in its nucleus and duplicates the centrosome. In the G2 phase, the cell grows more, makes proteins and organelles, and begins to reorganize its contents in preparation for mitosis. In the M phase, including mitosis and cytokinesis, the cell separates its DNA into two sets and divides its cytoplasm, forming two new cells.

Fig. 3 The cell cycle

Cancer cells behave differently than normal cells in the body. Many of these differences are related to cell division behavior. In contrast to normal cells, cancer cells may make their own growth factors, have growth factor pathways that are

always active or induce neighboring cells to produce growth factors to sustain them [64]. Cancer cells can also divide many more times than a normal cell and may ignore signals to stop dividing. Other hallmarks of cancer include resisting cell death, inducing angiogenesis, and activating invasion and metastasis [64, 65].

Oncogenes and Tumor suppressors

There are broadly two types of genes involved in tumor initiation and development of cancer: oncogenes and tumor suppressors. Many genes in these categories are involved in the regulation of cell division, cell death, DNA damage and repair. Whereas increased activity of oncogenes may induce cancer, tumor suppressors operate to reduce the likelihood of cancer. In many cancers, there is either a gain of function of oncogenes or loss of function of tumor suppressors which may induce the cell to go through multiple cell divisions and accumulate genetic defects.

Oncogenes

The normal form of an oncogene, that is not yet mutated, is called proto-oncogene. Several types of mutations may cause increased activity of the proto-oncogenes and turn them into oncogenes. This includes amplification, where a gene gains extra copies and starts producing too much of the protein that the gene is coding for. Other mutations change the amino acid sequence of the protein, which alters its shape and trapping it in a state where it is always active. In other cases, a proto-oncogene may attach to a part of a different gene (chromosomal translocation), whereby a new fusion gene is transcribed into a protein with dysregulated activity [31].

Many of the proteins that transmit growth factor signals are encoded by proto-oncogenes. Normally, these proteins drive cell cycle progression only when growth

factors are available. However, if one of the proteins becomes overactive due to mutation, it may transmit signals even when no growth factor is around. In breast cancer, *HER2* is a well-known oncogene due to gene amplification.

Tumor suppressors

Tumor suppressors are negative regulators of cell cycle progression. If they are not working properly, they may not sense signals of DNA damage or trigger a response. DNA methylation, which means that a methyl group is added to the DNA molecule, may change the activity of a gene without changing the DNA sequence. It is a common reason for silencing tumor suppressor genes. Two of four bases in the DNA, cytosine and adenine, can be methylated. Cytosine pairs with guanosin in CpG dinucleoties and regions with high frequency of CpG sites, so-called CpG islands, are common in promoter regions. Methylation can inhibit genes to be transcribed, either because transcription factors are impeded to bind to DNA, or that a protein complex which binds to the methyl groups alters the structure of histone proteins [66].

One of the most frequently mutated genes in cancer is the tumor suppressor p53, which controls the G1 to S transition. Cancer cells without p53 mutations likely inactivate p53 through other mechanisms [67].

If DNA damage occurs, a functional p53 acts in several ways. First, it blocks the cell cycle progression, gaining time to repair the damage. Secondly, it activates enzymes that may repair the damage. If this is successful the cell cycle may continue, otherwise p53 triggers apoptosis (programmed cell death) so that damaged DNA is not passed on [4, 68]. When p53 is defective, a cell with damaged DNA may proceed

with cell division. The daughter cells of such a division are likely to inherit mutations due to the unrepaired DNA of the mother cell.

RAB6C which was studied in paper III and IV in this thesis has been suggested as a tumor suppressor and is regulated by p53 [69, 70].

Signaling pathways

A signaling pathway is a group of proteins that work together to control cell functions, such as protein synthesis, cell division or cell death. A receptor receives a signal, like a hormone or growth factor, and passes this signal through the membrane onto other proteins, often due to phosphorylation. This means that a phosphate group is added to an amino acid of the next protein, which activates or inactivates the protein. The process is repeated until the last protein in the pathway is activated and the cell function is carried out. Abnormal activation of signaling pathways may lead to cancer. Therefore, drugs are being developed to block specific pathways.

The estrogen receptor (ER) and the progesterone receptor (PR)

Estrogen affects growth, differentiation and function of the mammary gland and mediates its effects by binding to its receptor, ER [71]. ERs are widely expressed in different tissue types, e.g. in the endometrium, breast, ovary and hypothalamus and belongs to the family of nuclear receptors, which are ligand-regulated transcription factors, activated by steroid hormones [72]. Two separate genes, *ESR1* and *ESR2*, encode the estrogen receptors ER α and ER β [73]. In this thesis, ER always refers to ER α .

Unlike most intercellular messengers, the ligands can cross the plasma membrane and directly interact with nuclear receptors inside the cell. In the absence of hormone, estrogen receptors are largely located in the cytosol. Hormone binding to the receptor triggers a number of events. The receptor migrates from the cytosol into the nucleus, pairs up with another receptor, forming a receptor dimer binding to specific sequences of DNA. Once activated, nuclear receptors directly regulate transcription of genes that control a wide variety of biological processes, including cell proliferation, development, metabolism, and reproduction [72, 74]. Besides direct binding to the DNA, ER may change various gene transcriptions indirectly, by binding to other transcription factors, such as AP-1, SP-1, STAT3, or NF- κ B [75-77]. Besides these so-called genomic mechanisms, ER may activate signal transduction pathways in the cytoplasm (non-genomic mechanism) [75, 78].

Fig. 4 The classical ER and PR signaling pathway

Approximately 75 - 85 % of the breast tumors have an overexpression of ER, i.e. are producing too much of this protein. They are classified as ER+, and of these, 75 - 85 %

are also PR+ [79]. If the tumor is PR+ it indicates an intact ER signaling pathway [80]. But absence of PR may have other reasons than a nonfunctional ER signaling pathway [81]. Growth factors in the insulin-like growth factor (IGF) and epidermal growth factor (EGF) families that activate the PI3K-AKT-mTOR pathway can decrease PR transcription even as they activate other ER functions [82, 83]. Several studies have shown that ER+/PR- tumors have higher levels of epidermal growth factor receptor (EGFR) and HER2 than ER+/PR+ tumors [82, 84, 85].

Tumors that are neither overexpressing ER nor PR (ER-/PR-) represent 10 - 20 % of the breast tumors and only a few percentages are classified as ER-/PR+. Tumors in the latter group are often claimed to be falsely classified as it is stated that PR may not be expressed in absence of ER. The presence of the subgroups differs among different age groups. Women younger than 40 years old are more often diagnosed with an ER-/PR- tumor and presence of ER+/PR- tumors is more common in the ages 55 – 65 [86-88].

The PI3K/AKT pathway

The phosphatidylinositol 3-kinase (PI3K) pathway is the most frequently mutated pathway in breast cancer. In over 70 % of the breast cancer cases there are mutations or gene copy number aberrations of the involved genes, e.g., HER2, PTEN, PIK3CA, IGF1R, FGFR1, RPS6K1, AKT1, AKT2 and EGFR [89]. Alterations in the pathway lead to changes of proteins which regulate cell cycle progression, cell proliferation and cell survival. Further, in ER+ breast cancer cells, PI3K activation promotes estrogen-dependent and -independent ER transcriptional activity, which, in turn, may contribute to anti-estrogen resistance [89, 90].

The PI3K/AKT pathway is activated by growth factor receptor tyrosine kinases (RTKs) and G-protein-coupled receptors (GPCRs). RTKs, for example HER2 and fibroblast

growth factor receptor (FGFR) 1, are enzymes that transfer phosphate groups to the amino acid tyrosine which hereby can transmit signals to other molecules in the cell. GPCRs are a family of cell surface receptors that transmit signals inside the cell through so called G proteins. G proteins bind the nucleotide guanosine triphosphate (GTP) and break it down to guanosine diphosphate (GDP), whereby phosphate is released and may activate signaling pathways. RTKs and GPCRs can recruit PI3K to the cell surface receptors, which results in production of 3'-phosphorylated phosphoinositide PIP₃. This further helps AKT to recruit to the membrane, where it is phosphorylated by phosphoinositide dependent kinase 1 (PDK-1) and mammalian target of rapamycin complex 2 (mTORC2) [91-94].

In turn, AKT can activate several proteins that regulate protein synthesis, cell proliferation and survival. PTEN acts as a negative regulator of the pathway by dephosphorylating PIP₃.

Fig. 5 The PI3K/mTOR pathway

AKT

AKT (also known as PKB) was first isolated in the 1970s from the transforming murine retrovirus AKT8 discovered in an AKR mouse thymoma cell line [95]. The human *AKT1* and *AKT2* genes could be cloned 10 years later, followed by a third *AKT* gene, *AKT3* in 1999 [96, 97]. The genes are located at different chromosomal locations, *AKT1* at 14q32, *AKT2* at 19q13 and *AKT3* at 1q44. The corresponding proteins show high homology, where AKT1 and AKT3 are most similar (83 %), followed by AKT1 and AKT2 (82 %) and AKT2 and AKT3 (77 %) [98]. Despite the similar structural domains, the proteins seem to have different functions. Studies of mammary tumors in mouse models have shown that whereas AKT1 plays an important role in tumor initiation, AKT2 is mainly involved in the metastatic process [99, 100]. AKT3 has been suggested to be involved in triple negative breast cancer, where targeting of AKT3 inhibited growth of triple-negative breast cancer cells in mouse models [98, 101]. Activating mutations in AKT1 are found in 2 % of all breast cancer tumors and belongs to the 20 most commonly mutated genes [102].

RAB6C

Rab-GTPases are members of the RAS family and their expression product can bind to GTP [69, 70, 103]. They are involved in regulating intracellular protein trafficking pathways and hypothesized to have altered expression levels in certain cancer [69]. One of five ancestral RAB genes is RAB6, which is conserved from yeast to humans. Gene duplication and alternative splicing during evolution has resulted in different *RAB6* isoforms in humans. *RAB6A* and the spliced variant *RAB6A'* are located on chromosome 11, *RAB6B* which is a gene duplication of *RAB6A/A'* on chromosome 3 and *RAB6C* (also known as *WTH3*) on chromosome 2. The *RAB6C* gene consists of a single exon and shares highest identity with the *RAB6A'* transcript. Therefore, it is suggested that *RAB6C* has been generated by retrotransposition of a fully processed *RAB6A'* mRNA, but encoding a functional protein with altered characteristics compared to *RAB6A'*.

Unlike other RAB6s, which are located in the Golgi apparatus, RAB6C is primarily distributed in the cytoplasm and in the nucleus [104]. The protein is involved in regulation of centrosome duplication and cell cycle progression [69]. As the *RAB6C* gene is directly regulated by the protein p53 it is suggested to promote apoptosis [105]. RAB6C has also been shown to be able to inhibit the proliferation of breast cancer cells and decrease their invasion ability [70].

Aims of the thesis

General aim

The aim of this thesis is to identify prognostic and predictive factors in ER+ breast cancer. In particular we focused on factors that influence the long-term survival, as patients with ER+ tumors often live long and have higher risk of late recurrences than those with ER- tumors.

Specific aims are

- To study the long-term prognosis related to AKT1 and AKT2 in ER+ breast cancer
- To study the role of PR in relation to tamoxifen benefit in ER+ breast cancer
- To identify new markers for subgroup division among patients with ER+/PR- tumors and specifically validate prognostic and predictive importance of RAB6C

Comments on material and methods

Patients

In all papers, data from a randomized trial conducted by the Stockholm Breast Cancer Study Group between 1976 and 1990 was used (Table 2). The patients were divided into a high-risk and a low-risk group, depending on the tumor size and lymph node status. For the low-risk patients, tamoxifen given post-operatively was compared with no adjuvant endocrine therapy. For the high-risk patients, chemotherapy was compared with RT. Data from the low-risk group was used for paper II, III, IV and data from the high-risk group for paper I and III. Vital status was collected from the Swedish Cause of Death Register. For paper III, we also used a public data set from National Center for Biotechnology Information (NCBI) with Dutch patients diagnosed between 1980 and 1995, who had node-negative, ER+ disease and did not receive adjuvant systemic therapy [106].

Ethical considerations

All procedures performed in the studies were in accordance with the ethical standards of the local ethical committee at the Karolinska University Hospital (KI 97-451 with amendment 030201) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Table 2 Patients included in the papers

Years of diagnosis	Inclusion criteria	Treatment	Paper
1976 - 1990	Premenopausal patients, T > 30 mm or N ≥ 1		
		RT	III
		Chemotherapy	-
1976 - 1990	Postmenopausal patients ≤ 70 years old, T > 30 mm or N ≥ 1		
		RT	I
		RT + Tamoxifen	I
		Chemotherapy	I
		Chemotherapy + Tamoxifen	I
1976 - 1990	"Low-risk" postmenopausal patients ≤ 70 years old, T ≤ 30 mm and N0		
		Tamoxifen	II & IV
		No tamoxifen	II & III & IV
		RT + Tamoxifen	II & IV
		RT	II & III & IV
1980 - 1995	N0, ER+		
		No adjuvant systemic therapy	III

The data material

The data from the Stockholm randomized trial has several advantages. It was one of the first randomized studies to compare tamoxifen treatment versus control, before it was known that only patients with ER+ tumors had substantial benefit from tamoxifen. Therefore, the data contains a large number of patients that are systemically untreated and, since the patients were recruited independently of ER status, both those with ER+ and ER- tumors. This makes it possible to analyze the natural course in relation to different factors in patients with either ER+ or ER- tumors. Furthermore, the follow-up time is long, over 20 years. On the other hand, the material has some limitations as it is old and the treatment has improved since then. The patients participating in the study received either two or five years of tamoxifen, whereas the endocrine treatment nowadays is given during at least five years.

Protein expression determination

Protein expression may be determined with different techniques, which have improved over time. ER assessment using cytosol assays have been compared with immunohistochemical staining (IHC) and was shown to provide similar results [107]. In our studies, we have used ER assessment as determined by IHC, while for tumors with missing data on ER by IHC, ER status according to the cytosol method was used. For PR assessment, the concordance between ligand binding assays and IHC is more uncertain. Previous studies have shown that PR assays have more variation and are less accurate than those for ER [108].

Cytosol assays

Until approximately 1990, ER and PR were determined with biochemical methods by which the level of the receptors was measured in cytosol. Before 1988, the content of ER and PR was determined using isoelectric focusing (IF) on polyacrylamide gel [109-111]. The tumor tissue was dissolved and mixed with radioactive estrogen which could bind to the receptors. Thereafter this mixture was put to migrate through a gel in order to separate the bounded protein from the rest. However, the receptors could be destroyed during the sensitive procedure and after 1988 it was replaced by an enzyme immunoassay (EIA). This method was more reliable since specific antibodies for the receptors were used [112-114]. The cytosol method was time-consuming, required large amounts of tumor material and ER and PR receptor content was measured in tumor tissue consisting of both cancer cells and stromal cells. However, an advantage of the method was the possibility of absolute quantification.

Immunohistochemical staining (IHC)

Generally, when IHC is used, the tumor tissue is fixed and unspecific binding sites are blocked before adding a primary antibody that binds to the target antigen in the tissue. Thereafter, a labeled secondary antibody that reacts with the primary antibody is used. Counterstaining and glass slide mounting is performed before evaluation.

IHC is a cheap, less time-consuming method for which formalin fixated paraffin embedded tissues may be used. Nowadays IHC is routinely used by pathologists for clinical evaluation of protein expression in tissues, e.g., ER and PR assessment. In this thesis we determined RAB6C expression using IHC. The possibility to determine subcellular location is an advantage of the method, but problems with reproducibility and lack of specific antibodies are challenging problems as well as subjective

determination. In order to reduce this problem, the IHC determinations in this thesis were performed blinded and of at least two independent observers.

Statistics

Subgroup analyses

The papers in the thesis include subgroup analyses. Such analyses should always be performed and interpreted with caution. If subgroups are created ad hoc, there is an extensive risk that at least one subgroup shows significant result even though this is not true (type I error). To avoid this problem, we have defined the subgroups in advance, based on well-known clinical subgroups. Another possibility to adjust for multiple analyses is to apply Bonferroni adjustment, though it may be claimed to be too conservative. One should also be aware of the fact that subgroup-specific analyses are affected by many factors and should rather be based on formal tests of interactions. An interaction describes a situation where the effect of two independent variables on a third is not additive, i.e. the effect of one independent variable may depend on the level of the other independent variable. Though, most studies are underpowered for detecting interaction effects [115-118].

Survival analysis

Survival curves were estimated using the Kaplan-Meier approach, which provides a nonparametric estimate of the survival probability of an event of interest [119].

In order to relate risk factors (covariates) to survival time, we applied Cox proportional hazard regression analysis [120]. The model is called semi-parametric, since it is non-parametric with respect to time but parametric in terms of the covariates. The hazard rate refers to the instantaneous rate of failure (for example

recurrence or death) during the next instant of time, given that the patient has survived until a specific time. When we compare two independent groups, we estimate the hazard ratio (HR). This is the ratio of the hazard rates between the two independent comparison groups [121]. The HR estimates how many times higher or lower the risk of event is for one group compared with the reference group. If HR exceeds 1, it means a higher risk of event and HR less than 1 means a lower risk of event. HR is reported with a 95 % confidence interval (CI), which means that if we were to draw several independent, random samples from the same population and calculate 95 % CI from each of them, then on average 95 % such CI would contain the true population HR [122]. The p-values are obtained from two-sided Wald tests. For appropriate use of Cox proportional hazard regression model, some assumptions must be fulfilled:

- a) the hazard ratio is constant over time (proportional hazards)
- b) independent censoring (i.e. the items may not be censored because they have a higher or lower risk than the average, given the covariates)
- c) a multiplicative relationship between the covariates and the hazard

Subpopulation treatment effect pattern plot (STEPP)

STEPP is an exploratory technique for graphical testing of interactions between treatment and continuous covariates [123]. It considers sequences of potentially overlapping subpopulations defined with respect to a covariate and is especially useful when trying to identify subpopulations of patients for whom the overall trial results may be less representative. There are several advantages of the method, e.g., that no assumptions about the nature of the relationship between the outcome and the covariate in each treatment group must be done and that it avoids dichotomization of covariates, which may be subjective. Though, the size of the subpopulations is critical to the performance of the method and, hence, to the interpretation of the results [124, 125]. In paper II, we used STEPP to investigate the interaction between continuous PR values and the effect of tamoxifen. To avoid

results based on one single subgroup division, we performed the STEPP analysis with different numbers of subgroups and ensured that the achieved results were similar independently of the chosen number of subgroups.

SNP selection among whole genome gene copy data

In paper III, we used a public data set including whole genome gene copy data of 199 patients. We defined the most varying single-nucleotide polymorphism (SNPs) as those with the largest interquartile range (IQR), i.e. the SNPs where the middle 50 % has the largest variation among the patients. For the 20 most varying SNPs, the patients were divided into two groups with the median value of gene copies as cut-off. In order to investigate the prognostic value of each of the 20 SNP separately, the cumulative distant-recurrence risk was plotted and HRs with 95 % CI were estimated.

The selecting approach using IQR implies that we select SNPs that vary a lot for relatively large groups of patients, whereas we do not identify rare SNPs with large variation. For example, the *HER2* gene, which is known to be amplified among about 15 % of the patients, did not come up among the top varying SNPs. On the other hand, by excluding the upper and lower 25 % of the values, we do not risk to deal with outliers. The approach to compute the IQR for SNPs has been applied previously [126].

The selected number of SNPs and the fact that we choose the middle 50 % may be questioned. For example, choosing the middle 90 % instead would result in other top varying SNPs. Furthermore, SNPs that are nearby are highly correlated, implying that there is a high risk that several SNPs that are located close to each other are chosen. One strength of our method is that it is a simple approach to filter out SNPs with consistently low values or low variance across the samples. Performing this method, we identified the gene *RAB6C* as a prognostic marker. The statistical finding of *RAB6C* as an interesting prognostic factor was supported by the literature, which brought us to perform further experiments on the protein RAB6C [69, 70, 104, 105, 127-130].

Results and discussion

Paper I

Besides conventional prognostic factors, such as nodal status and tumor size, several biomarkers are claimed to provide prognostic information, but many of them need further follow-up to confirm long-term effects [131]. In paper I, we revealed the different roles of AKT1 and AKT2 and their long-term significance in relation to prognosis. We showed that for patients with ER+ tumors, AKT2 is an independent prognostic factor for distant recurrence-free survival. In contrast to many well-known prognostic factors, the significance of AKT2 remained several years after diagnosis. In a multivariable analysis for ER+ tumors including established clinical factors, the risk reduction was 62% for patients with tumors expressing AKT2 (AKT2+) compared with those which did not express AKT2 (AKT2-) (HR = 0.38, 95% CI 0.21–0.68, $p = 0.001$). For patients with ER- tumors there was no statistical significant difference in distant recurrence-free survival (HR = 1.24, 95% CI 0.56 – 2.73, $p = 0.59$). For patients with tumors expressing AKT1 (AKT1+) we could not show prolonged or shortened distant recurrence-free survival compared with patients whose tumors did not express AKT1 (AKT1-), irrespective of ER status: ER- tumors, HR= 0.98 (95% CI 0.50 – 1.91, $p = 0.95$); ER+ tumors, HR= 0.89 (95% CI 0.58 – 1.38, $p = 0.61$).

In order to investigate whether AKT1 or AKT2 has a long-term significance on prognosis for patients with ER+ tumors, we performed a multivariable analysis dividing the follow-up period into two different time intervals, before and after 5 years since diagnosis. While tumor size failed to provide long-term prognostic information, AKT2 remained statistically significant even after 5 years (HR= 0.43, 95% CI 0.19 – 0.97, $p = 0.043$). For AKT1, there was no statistical significance and the HR was approximately 1 independently of time period (Table 3).

Table 3 Multivariable analysis of distant recurrence-free survival for patients with ER+ tumors by follow-up period^a

	<5 years		≥5 years	
	HR	95% CI	HR	95% CI
AKT1 (pos. vs. neg.)	1.08	0.55 – 2.10	1.03	0.50 – 2.13
AKT2 (pos. vs. neg.)	0.32*	0.13 – 0.78	0.43*	0.19 – 0.97
pAKT ^b (pos. vs. neg.)	0.94	0.52 – 1.70	1.00	0.51 – 1.98
Tumor size (>20 vs. ≤20)	2.09*	1.12 – 3.87	1.22	0.59 – 2.52
Nodal status (1-3 vs. 0)	3.11	0.70 – 13.7	1.33	0.40 – 4.46
(4- vs. 0)	4.64*	1.02 – 21.0	3.94*	1.08 – 14.4
HER2 (pos. vs. neg.)	1.14	0.59 – 2.19	1.22	0.52 – 2.87
PIK3CA ^c (mutated vs. wt ^d)	1.06	0.55 – 2.02	1.09	0.53 – 2.25
Tamoxifen (yes vs. no)	0.41**	0.22 – 0.76	0.54	0.28 – 1.07
CMF ^e (yes vs. no)	0.87	0.49 – 1.55	0.89	0.46 – 1.72

*p< 0.05, ** p< 0.01

a) Multivariable analysis was performed for patients where we had data on all variables included (163 patients for the first time period and 110 patients for the second time period)

b) pAKT= activated AKT

c) PIK3CA= phosphatidylinositol 3-kinase catalytic subunit alpha

d) wt= wildtype

e) CMF= cyclophosphamide, methotrexate and fluorouracil

The ER+ tumors were further divided into two groups with intermediate levels of ER (between 0.1 and 1.0 fmol/μg DNA) and high levels (≥1.0 fmol/μg DNA). A test for trend showed that the prognostic significance of AKT2 increased with higher estrogen receptor expression from HR=1.00 (95% CI 0.53 – 1.87, p= 1.00) for patients with ER- tumors to HR= 0.67 (95% CI 0.35 – 1.27, p= 0.22) for intermediate levels of ER to HR= 0.32 (95% CI 0.14 – 0.77, p= 0.010) for high levels of ER (P for trend= 0.042) (Fig. 6 and Fig. 7).

Fig. 6 Distant recurrence-free survival in relation to AKT2 for patients with ER+ tumors, HR= 0.49 (95% CI 0.29 – 0.82, $p=0.007$) **(a)** and ER- tumors, HR=1.00 (95% CI 0.53 – 1.87, $p=1.00$) **(b)**

Fig. 7 Distant recurrence-free survival in relation to AKT2 for patients with tumors with intermediate ER-levels ($0.1 \leq ER < 1.0$ fmol/ μ g DNA), HR= 0.67 (95% CI 0.35 – 1.27, $p=0.22$) **(a)** and high ER-levels (≥ 1.0 fmol/ μ g DNA), HR= 0.32 (95% CI 0.14 – 0.77, $p=0.010$) **(b)**

The better prognosis for patients with ER+ tumors and AKT2 expression was more pronounced in the combination of AKT2+ and activated AKT (pAKT+) (HR= 0.31, 95% CI 0.13 – 0.73, $p= 0.007$) compared with non-activated AKT (pAKT-) (HR= 0.71, 95% CI 0.37 – 1.37, $p= 0.30$) (Fig. 8) A test for interaction between ER status and the combined AKT2/pAKT status showed statistical significance ($p= 0.015$) with respect to prognosis.

Fig. 8 Distant recurrence-free survival in relation to AKT2 for patients with ER+/pAKT+ tumors, HR= 0.31 (95% CI 0.13 – 0.73, $p= 0.007$) **(a)** and ER+/pAKT- tumors, HR= 0.71 (95% CI 0.37 – 1.37, $p= 0.30$) **(b)**

The specific functions of AKT1 and AKT2 are mainly due to partly different subcellular locations and interactions with different proteins [99]. Studies about the prognostic value of AKT show partly different results [98]. One reason may be that the antibodies could not distinguish between the isoforms, and since they act in different ways, they may show contradictive results. Probably, isoform specific inhibitors are needed, though the high homology may cause a problem in developing such inhibitors.

Paper II

The clinical value of PR status has been discussed in various studies [80, 81, 83, 132, 133]. One argument against PR determination has been that PR has very little therapeutic impact since more than 99 % of the ER- patients are also PR-, and ER+ patients receive endocrine therapy independently of PR status [132]. On the other hand, the St Gallen Criteria emphasize the significance of PR status for the oncologic therapy, where ER+/PR- are recommended a more aggressive treatment than ER+/PR+ [40]. In paper II, we aimed to rule out the predictive value of PR (determined by IHC) in relation to tamoxifen benefit, in particular the long-term effect. We evaluated 618 patients with ER+ "low-risk" breast cancer (size ≤ 30 mm, lymph node-negative) for whom PR was determined by IHC at one pathology laboratory. PR was defined as PR+ if there was ≥ 10 % stained cells.

Our results showed that tamoxifen had slightly more effect on the recurrence risk for patients with ER+/PR+ than ER+/PR- tumors the first five years ER+/PR+: HR= 0.27 (95 % CI: 0.14 – 0.53), $p < 0.001$, ER+/PR-: HR= 0.39 (95 % CI: 0.15 – 1.00), $p = 0.05$.

Thereafter, the relative risk ratio increased more for patients with ER+/PR- tumors (HR after 5 years= 1.34 (95 % CI: 0.69 – 2.60, $p = 0.39$) compared with ER+/PR+ (HR after 5 years= 0.55 (95 % CI: 0.34 – 0.88, $p = 0.012$). (Fig. 9)

Fig. 9 Cumulative recurrence risk for patients with ER+/PR+ tumors, HR= 0.43 (95% CI 0.29 – 0.62, $p < 0.001$) **(a)** and ER+/PR- tumors, HR= 0.87 (95% CI 0.52 – 1.46, $p = 0.59$) **(b)**

We further investigated how the tamoxifen effect varied over different levels of PR, both using subgroups based on cut-offs at 1 %, 10 %, 50 % and 75 % stained cells, as well as performing STEPP analysis where previously determined cut-off values are not needed. The results from both analyses showed that at all receptor levels with $\geq 10\%$ stained cells there was a benefit from tamoxifen (Fig. 10 and Table 4). The STEPP analysis also indicated some effect of tamoxifen even in patients with ER+/PR- tumors the first five years after diagnosis, which is in line with the results in Table 4 (Fig. 11).

Fig. 10 Influence of increasing proportion of PR-positive tumor cells on cumulative recurrence risk among patients with ER-positive tumors. PR 1 - 9 %, HR= 1.11 (95% CI 0.38 – 3.24), $p= 0.84$ **(a)**, PR 10 – 49 %, HR= 0.30 (95% CI 0.16 – 0.58), $p< 0.001$ **(b)**, PR 50 – 74 %, HR= 0.38 (95% CI 0.18 – 0.80), $p= 0.011$ **(c)**, PR $\geq 75 \%$, HR=0.59 (95% CI 0.32 – 1.08), $p= 0.086$ **(d)**

Table 4 Outcome for patients with ER positive tumors divided by different levels of PR (IHC) expression and different time periods

Tam vs. control	PR (IHC)	0 – 5 years				Beyond 5 years			
		Number of Patients/Events		HR (95 % CI)	P value	Number of Patients/Events		HR (95% CI)	P value
		TAM	Control			TAM	Control		
Recurrence rate	0 %	79/4	82/13	0.29 (0.09 – 0.89)	0.031	73/15	64/11	1.34 (0.62 – 2.93)	0.46
	1 - 9 %	25/2	25/2	1.07 (0.15 – 7.56)	0.95	22/6	21/4	1.14 (0.32 – 4.05)	0.85
	10 - 49 %	70/8	50/12	0.44 (0.18 – 1.07)	0.069	59/6	37/15	0.21 (0.08 – 0.54)	0.001
	50 - 74 %	63/2	52/10	0.15 (0.03 – 0.69)	0.015	55/9	40/10	0.59 (0.24 – 1.46)	0.26
	≥ 75 %	92/2	80/11	0.15 (0.03 – 0.67)	0.013	85/16	66/14	0.93 (0.45 – 1.91)	0.85

Fig. 11 Subpopulation treatment effect pattern plots (STEPP), showing the effect of tamoxifen vs. control on the recurrence risk during the first five years after diagnosis **(a)**, more than five years after diagnosis **(b)** in relation to PR values measured with IHC. HR (solid black line) with the corresponding 95% confidence interval (dashed grey lines) is plotted against the mean PR for overlapping subgroups. The dotted black line shows the HR for tamoxifen vs. control for all PR values in the selected time period.

Our results are partially contradictory to the data in the Early Breast Cancer Trialists' Collaborative Group (EBCTCG) review where PR did not add predictive value for tamoxifen to ER alone [134]. As explained below, we suggest three possible reasons for the contradictory results: a) the method of PR determination, b) a time-dependent effect and c) use of chemotherapy.

a) Most PR values in the EBCTCG overview were obtained with cytosol based assays. In our cohort, cytosol PR information was also available and in agreement with the EBCTCG review, PR positivity determined with cytosol assay did not predict tamoxifen benefit more efficiently than ER positivity alone ER+/PR+: HR= 0.45 (0.28 – 0.72, p= 0.001), ER+/PR-: HR= 0.48 (0.27 – 0.87, p= 0.015). In the EBCTCG overview it is stated that 21% of the ER-negative cancers were PR positive, indicating that the PR technique used resulted in many false positive PR classifications.

b) As the follow-up period in the present study was long and the predictive value for PR was most evident beyond 5 years of follow-up, it may indicate a time-dependence of the ability of PR to predict the efficacy of adjuvant tamoxifen.

c) The patients in the present study did not receive adjuvant chemotherapy. Potential differences in tamoxifen benefit in relation to PR levels could possibly be masked by the effect of chemotherapy, in particular in cohorts where the use of chemotherapy is not balanced between patients with ER+/PR+ and ER+/PR- tumors.

Paper III

The aim of paper III was to identify new prognostic factors among patients with ER+ tumors. We specifically aimed to investigate if these factors have prognostic value in subgroups based on PR. Using the public data set with accession number *GSE10099* including whole genome gene copy data, available at the National Center for Biotechnology Information (NCBI) website, we identified one candidate gene, *RAB6C*, for subgroup division among patients with ER+/PR- tumors. Our results suggested that if there were few gene copies of *RAB6C*, it was related to poor prognosis. For patients with ER+/PR- tumors, there was a risk reduction of 77 % (HR= 0.23, 95% CI: 0.08 – 0.61, $p = 0.003$) if the tumor had gene copies above the median value (*RAB6C*+) compared with below (*RAB6C*-). Even for patients with ER+/PR+ tumors, *RAB6C* had a favorable prognostic value, though it was associated with less risk reduction (HR= 0.55, 95% CI: 0.32 – 0.96, $p = 0.03$) (Fig. 12 and Table 5).

Fig. 12 Cumulative distant recurrence risk in relation to gene copy number of *RAB6C* for patients with ER+/PR-, HR= 0.23 (95 % CI: 0.08 – 0.61), $p = 0.003$ **(a)** and ER+/PR+, HR= 0.55 (95 % CI: 0.32 – 0.96), $p = 0.03$ **(b)** tumors in the public data set

Table 5 Distant recurrence rate in the public data set for *RAB6C*⁺ compared with *RAB6C*⁻ divided by hormone receptor status. *RAB6C*⁻ is the referent (with HR= 1) in each subgroup analysis.

Hormone receptor status	Number of Patients/Events		Univariable		Multivariable*	
	<i>RAB6C</i> ⁺	<i>RAB6C</i> ⁻	HR (95 % CI)	P value	HR (95 % CI)	P value
ER+	99/28	100/50	0.44 (0.28 – 0.71)	0.001	0.45 (0.28 – 0.72)	0.001
ER+/PR+	73/22	69/31	0.55 (0.32 – 0.96)	0.034	0.55 (0.32 – 0.95)	0.033
ER+/PR-	23/5	29/19	0.23 (0.08 – 0.61)	0.003	0.15 (0.05 – 0.46)	0.001

*Multivariable analysis for ER+ tumors adjusted for age, tumor stage, grade and PR. The multivariable analysis confined to the subgroups ER+/PR+ and ER+/PR- adjusted for age, tumor stage and grade

We also determined the protein expression of RAB6C in an independent cohort with similar results. High expression of RAB6C (*RAB6C*⁺) compared with low expression (*RAB6C*⁻) among patients with ER+/PR- tumors implied better prognosis (HR= 0.24, 95% CI: 0.09 – 0.66, *p* = 0.005) whereas we could not show statistical significance for patients with ER+/PR+ tumors (HR= 1.20, 95% CI: 0.66 – 2.19, *p*=0.55) (Fig. 13). Adjusting for age, tumor size, lymph node status, HER2, grade, and PR in a multivariable analysis including patients with ER+ tumors, RAB6C retained its prognostic importance, which was stronger for patients with ER+/PR- tumors as indicated by a statistically significant interaction between PR and RAB6C (*p*= 0.005). Stratifying the results of the multivariable analysis according to PR status, the distant recurrence rate for *RAB6C*⁺ versus *RAB6C*⁻ was clearly reduced among patients with ER+/PR- tumors (HR= 0.17, 95% CI: 0.05 – 0.60, *p* = 0.006), but not in patients with ER+/PR+ tumors (HR= 1.31, 95% CI 0.69 – 2.48, *p*= 0.41) (Table 6).

Fig. 13 Cumulative distant recurrence risk in relation to protein expression of RAB6C for patients with ER+/PR-, HR= 0.24 (95% CI: 0.09 – 0.66), $p=0.005$ (a) and ER+/PR+, HR= 1.20 (95% CI: 0.66 – 2.19), $p=0.55$ (b) tumors in the independent cohort

Table 6 Distant recurrence rate in the independent cohort for RAB6C+ compared with RAB6C- divided by hormone receptor status. RAB6C- is the referent (with HR= 1) in each subgroup analysis.

Hormone receptor status	Number of Patients/Events		Univariable		Multivariable*	
	RAB6C+	RAB6C-	HR (95 % CI)	P value	HR (95 % CI)	P value
All	204/49	265/73	0.82 (0.57 – 1.18)	0.29		
ER+	153/32	157/40	0.77 (0.48 – 1.22)	0.27		
ER-	30/13	68/23	1.30 (0.66 – 2.56)	0.45		
ER+/PR+	87/23	92/20	1.20 (0.66 – 2.19)	0.55	1.31 (0.69 – 2.48)	0.41
ER+/PR-	51/5	50/17	0.24 (0.09 – 0.66)	0.005	0.17 (0.05 – 0.60)	0.006

*Multivariable analysis confined to ER+ tumors, including age, tumor size, lymph node status, HER2, grade, PR and the interaction term PR x RAB6C

In summary, at the first step, we analyzed whole genome gene copy data. By a statistic approach and support from the literature, which suggests interesting properties of RAB6C, this gene was selected and the protein expression was further analyzed in an independent cohort [69, 70, 104, 105, 127-130]. The prognostic value of RAB6C was significant both at gene copy level and protein expression level. RAB6C has been shown to serve as a useful biomarker and to have prognostic significance even among other cancer forms [135, 136]. An interesting aim for future experimental studies is to rule out the function of the protein.

Paper IV

In paper II, we showed that patients with ER+/PR+ tumors clearly did benefit from tamoxifen, while the long-term benefit was less for those with ER+/PR- tumors. However, patients with ER+/PR- tumors are a heterogeneous group and among them there might be subgroups of patients that do benefit from tamoxifen. The results from paper III indicated that RAB6C is a prognostic marker for subgroup division of ER+/PR- tumors. Therefore, the aim of paper IV was to investigate if RAB6C has a treatment predictive value for tamoxifen. Patients with ER+ tumors did benefit from tamoxifen and it was most evident among those with ER+/PR+ tumors (HR= 0.39, 95 % CI: 0.23 – 0.67, p= 0.001) compared with those with ER+/PR- tumors (HR= 0.65, 95 % CI: 0.34 – 1.25, p= 0.19). For the ER+/PR+ subgroup, patients with either RAB6C+ or RAB6C- tumors did benefit from tamoxifen, although it was statistical significant only among RAB6C+: HR (RAB6C+)= 0.27 (95 % CI: 0.13 – 0.58, p= 0.001) and HR (RAB6C-)= 0.63, 95 % CI: 0.29 – 1.37, p= 0.24), respectively. Among patients with ER+/PR- tumors, we could only show a statistical significant tamoxifen benefit for RAB6C- with HR= 0.25 (95 % CI: 0.09 – 0.70, p= 0.008) whereas the point estimate for RAB6C+ even was in the opposite direction with HR= 1.82 (95 % CI: 0.69 – 4.79, p= 0.23) (Table 7 and Fig. 14). The statistical significant interaction (p= 0.004) also indicated a different effect of tamoxifen among ER+/PR- tumors depending on RAB6C status.

Table 7 Univariable analysis of distant recurrence rates for tamoxifen (TAM) treated patients compared with the control group divided by hormone receptor status. The control group is the referent (with HR=1) in each subgroup analysis.

Subgroup	Number of Patients/Events		HR (95 % CI)	P value	P for interaction
	TAM+	TAM-			
ER+	259/35	227/61	0.47 (0.31– 0.71)	< 0.001	
ER+/PR+	177/20	143/39	0.39 (0.23 – 0.67)	0.001	0.20
ER+/PR-	82/15	84/22	0.65 (0.34 – 1.25)	0.19	
ER+/PR+/RAB6C+	111/10	75/22	0.27 (0.13 – 0.58)	0.001	0.14
ER+/PR+/RAB6C-	66/10	68/17	0.63 (0.29 – 1.37)	0.24	
ER+/PR-/RAB6C+	37/10	41/7	1.82 (0.69 – 4.79)	0.23	0.004
ER+/PR-/RAB6C-	45/5	43/15	0.25 (0.09 – 0.70)	0.008

Fig. 14 Distant recurrence-free survival in relation to tamoxifen (TAM) for patients with ER+/PR-/RAB6C+, HR= 1.82 (95 % CI 0.69 – 4.79), p= 0.23 **(a)** ER+/PR-/RAB6C-, HR=0.25 (95 % CI 0.09 – 0.70), p= 0.008 **(b)** ER+/PR+/RAB6C+, HR= 0.27 (95 % CI 0.13 – 0.58), p= 0.001 **(c)** ER+/PR+/RAB6C-, HR= 0.63 (95 % CI 0.29 – 1.37), p= 0.24 **(d)**

Among patients with ER+/PR- tumors, more specific subgroups are needed and our results may contribute to identification of such subgroups. The results suggest that patients with ER+/PR- tumors and low RAB6C expression do benefit from tamoxifen, whereas this could not be shown for those with high RAB6C expression.

General discussion and clinical implications

Patients with ER+ tumors have substantial long-term recurrence risk, despite five years of endocrine treatment [45]. Prolonged endocrine treatment has been suggested, but one question to be addressed is how to distinguish between patients that benefit from more treatment and patients with good survival anyhow.

Therefore, more specific long-term prognostic and predictive factors are needed. In this thesis, we aimed to identify factors that contribute to rule out this question.

In short, we suggest AKT2 as a long-term prognostic factor, PR as a long-term predictive factor and RAB6C as a marker for subgroup division among ER+/PR- tumors.

In order to study long-term effects, we used an old data material from a study with patients being randomized between 1976 and 1990. In paper I and II, we subdivided the data into different time periods as the aim was to focus specifically on the long-term risk. The data material was too small for such a subdivision in paper III and IV without getting statistical power problem.

Our results may contribute to identification of more specific subgroups regarding treatment determination, in particular for patients with stage I disease, which is the largest group of patients and that are generally considered to have good 5-year survival. In the future, some stage I patients with ER+ tumors may be recommended prolonged or reduced treatment, depending on the status of the tumor markers PR and RAB6C. The summarized results suggest that approximately half of the patients with ER+/PR- tumors do benefit from tamoxifen (Table 8).

Table 8 Patient distribution and survival in relation to the tumor markers PR and RAB6C

	PR+	PR-
RAB6C+	~ 40 % of the patients with ER+ tumors Good survival with TAM	~ 15 % of the patients with ER+ tumors Good survival without TAM
RAB6C-	~ 30 % of the patients with ER+ tumors Good survival with TAM	~ 15 % of the patients with ER+ tumors Poor survival without TAM Good survival with TAM

In this thesis, we investigated the clinical value of AKT2 in stage II breast cancer. In future studies, it would be interesting to include stage I patients. Another interesting aspect for future studies is to investigate the clinical behavior of AKT2 in relation to PR status.

In our studies, RAB6C was shown to provide both prognostic and predictive information about the patients’ outcome, but the knowledge about the biological function of the protein is limited. Therefore, it would be interesting to perform further experimental studies in order to understand the function.

Conclusions

- AKT2 expression is associated with a lower distant recurrence rate for patients with ER+ tumors and this association remains long-term. The prognostic significance of AKT2 increases with higher estrogen receptor expression. We could not show statistical significance of AKT1 expression.
- PR status determined by IHC adds predictive value to ER considering the long-term benefit from tamoxifen.
- Among systemically untreated patients with ER+/PR- tumors, RAB6C independently prognosticates the risk of distant recurrence.
- Patients with ER+/PR- tumors and low RAB6C expression do benefit from tamoxifen.

Acknowledgments

Jag vill tacka alla som har bidragit till detta arbete på olika sätt, i stort och smått. Tillsammans har ni gjort det möjligt för mig att genomföra arbetet med avhandlingen. Jag riktar ett stort tack till:

Min **huvudhandledare Olle Stål** och min **bihandledare John Carstensen**. Jag är djupt imponerad av er kunskap och av att ni är så snabba i tanken. Det är alltid intressant och roligt att diskutera med er. Jag kan inte tänka mig bättre handledare än er två och jag ser alltid fram emot våra möten i "Grodan".

Alla medförfattare till arbetena i denna avhandling och i andra forskningssamarbeten.

Tack för det fina samarbetet, för att ni lär mig mycket och bidrar med så bra idéer. Speciellt tack till:

Bo Nordenskjöld för att du har enorm kunskap om bröstcancer och alltid visar stort intresse för mina arbeten. Och tack för alla humoristiska, kvicka kommentarer.

Anna Nordenskjöld för gott samarbete i arbete 2 i avhandlingen och i andra gemensamma forskningsarbeten.

Lars-Gunnar Arnesson för att det är så roligt att jobba med dig och för att jag har fått lära mig mycket om bröstcancer genom våra samarbeten över åren.

Alla nuvarande och tidigare medarbetare på Regionalt Cancercentrum Sydöst för att ni gör det roligt att gå till jobbet, trots det tunga ämnet och för att ni under perioder har avlastat mig i mitt arbete, så att jag har kunnat frigöra tid för att skriva klart avhandlingen. Jag vill också tacka mina **verksamhetschefer Hans Starkhammar** och **Srinivas Uppugunduri** för att ni har låtit mig avsätta en del av arbetstiden till doktorandstudierna.

Alla nuvarande och tidigare medarbetare i Olle Ståls forskargrupp för att ni ofta kommer med nya infallsvinklar och kloka synpunkter. Tack för att jag har fått sitta och jobba hos er i perioder trots att jag som statistiker är en "outsider". Det har varit så trevligt! **Cynthia Veenstra**, tack för din språkliga expertis. Och tack för allt labb-arbete med

Stockholmsstudierna (**Birgitta Holmlund**), AKT (**Gizeh Pérez-Tenorio**) och RAB6C (**Josefine Bostner och Tove Bekkhus**).

Ämnesföreläsare Charlotta Dabrosin för att jag har fått möjlighet att genomföra min forskarutbildning inom onkologi vid Linköpings universitet.

Anand Srivastav. Thank you for inviting me to write my master thesis in your research group at Christian-Albrechts-Universität in Kiel. I had a great time in Kiel and it caught my interest in cancer research.

Alla patienter som deltar i forskningsstudier och låter forskare få tillgång till era data. Tack vare er kan cancerbehandlingen förbättras för framtida patienter.

All vårdpersonal som jobbar med cancerpatienter. Ni gör ett fantastiskt jobb med all er kunskap och goda omvårdnad!

Vänner och släktingar för alla gånger ni får mig att koppla av och bara ha det bra. Dessa stunder är så värdefulla!

Min barndomsvän Linda för allt vi har upplevt tillsammans (förtroliga samtal, skratt och tokiga resor) från att vi var små barn och in i vuxenlivet. Och tack för det fina omslaget till avhandlingen!

Min familj, som jag är så stolt över. Tack för allt vi delar i livet.

Mamma och pappa, världens bästa föräldrar och morföräldrar! Tack för tryggheten ni ger mig. Ni ställer upp så mycket och finns där för mig när jag behöver det! Tack vare er har jag klarat av flera hinder på vägen.

Min syster Maria för all uppmuntran och allt stöd under hela min doktorandtid. Men framför allt tack för all din omtanke, roliga telefonsamtal och allt annat du är och gör som storasyster och vän.

Min syster Johanna, Joel och Elliot för alla fina samtal, för att vi kan skratta och gråta ihop och för att det alltid är härligt att träffa er. Tack för att ni finns! Jag är så glad över att vara moster till fina Elliot.

Min dotter Linnéa. Min stjärna. För att du är det bästa i mitt liv. För att din betydelse inte går att beskriva med ord.

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