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Prognostic and Treatment Predictive Markers Associated with Cyclin D1 Gene Amplification and Epithelial-Mesenchymal Transition in Breast Cancer

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Prognostic and Treatment Predictive Markers
Associated with Cyclin D1 Gene Amplification and
Epithelial-Mesenchymal Transition in Breast Cancer

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Prognostic and Treatment Predictive Markers Associated with Cyclin D1 Gene Amplification and Epithelial-Mesenchymal Transition in Breast Cancer

Katja Lundgren



LUND UNIVERSITY
Faculty of Medicine

Academic Dissertation

By due permission of the Faculty of Medicine, Lund University, Sweden,
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Abstract <p>Endocrine resistant breast cancer is a major therapeutical challenge and accurate selection of patients susceptible to certain types of anti-hormonal treatment strategies is pivotal. Discovery of novel biomarkers potentially predicting treatment response as well as the clinical course of breast cancer is essential to effectively improve therapies and diagnosis.</p> <p>The main goal of this thesis was to identify putative markers for prediction of clinical outcome and response to tamoxifen, the first-line endocrine treatment option for premenopausal breast cancer patients. The markers examined were associated with CCND1 amplification (Chk1 and β-arrestin1), which has previously been linked to a potential adverse effect of tamoxifen, or with epithelial-mesenchymal transition (Snail), a biological process crucial for the development of metastasis.</p> <p>Immunohistochemical analyses of the biomarkers were predominantly performed in a breast cancer cohort including premenopausal patients randomly assigned to receive either tamoxifen or no adjuvant treatment. The in vitro studies investigated the regulation of EMT in hypoxia, in a panel of human breast cancer cells lines. We found that deletion of the CHK1 gene is potentially associated with CCND1 amplification, and loss of Chk1 protein expression was demonstrated to be linked to an impaired response to tamoxifen in premenopausal breast cancer patients.</p> <p>Additionally, the EMT-regulator Snail, which has been reported to be involved in ER- signaling, was shown to predict tamoxifen response, where absence of nuclear Snail rendered patients less sensitive to this adjuvant therapy. Moreover, hypoxia induced an incomplete EMT in vitro, and Snail modulated the migratory propensity of breast cancer cells.</p> <p>Finally, stromal expression of β-arrestin1 was found to be an independent prognostic marker in a cohort of both pre- and postmenopausal breast cancer patients. In premenopausal patients both high expression and absence of β-arrestin1 was linked to poor prognosis, but expression of this protein was not associated with an altered tamoxifen response.</p> <p>In conclusion, these studies revealed two biomarkers with potential clinical significance in predicting the sensitivity to adjuvant tamoxifen, as well as one with an impact on clinical outcome.</p>		
Key words: Breast cancer, tamoxifen, prognostic/predictive marker, cyclin D1 gene amplification, Chk1, β -arrestin1, EMT, Snail, hypoxia		
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Signature Katja Lundgren

Date 2010-02-25

For my Parents

*"Whatever you do will be insignificant,
but it is very important that you do it"*

-Mahatma Gandhi

List of Papers

This thesis is based on the following papers, referred to in the text by their respective roman numerals.

Paper I

Lundgren K, Holm K, Nordenskjöld B, Borg Å, Landberg G.
Gene products of chromosome 11q and their association with *CCND1* gene amplification and tamoxifen resistance in premenopausal breast cancer.
Breast Cancer Research: 10(5):R81 (2008)

Paper II

Lundgren K, Nordenskjöld B, Landberg G.
Hypoxia, Snail and incomplete epithelial-mesenchymal transition in breast cancer
British Journal of Cancer: 101(10):1769-81 (2009)

Paper III

Lundgren K, Tobin N P, Stål O, Rydén L, Jirstrom K, Landberg G.
Stromal expression of β -arrestin1 predicts clinical outcome in breast cancer
Submitted for publication

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Lundgren K, Holm C, Landberg G.
Hypoxia and breast cancer: prognostic and therapeutic implications. Review
Cellular and Molecular Life Sciences: 64(24): 3233-47 (2007)

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Abbreviations

AF-1/2	Activation function-1/2	GSK-3 β	Glycogen synthase kinase-3 β
AI	Aromatase inhibitor	HER2	Human epidermal growth factor receptor 2
AIB1	Amplified in breast cancer 1	HIF	Hypoxia inducible factor
AP-1	Activating protein-1	HPA	Human protein atlas
ARNT	Aryl hydrocarbon receptor nuclear translocator	HRE	Hypoxia response element
ARRB1	β -arrestin1 (gene)	HSC/HPC	Hematopoietic stem cell/progenitor cell
ATM	ataxia telangiectasia mutated	IGF-1R	Insulin-like growth factor-1 receptor
ATR	ATM and Rad related	IHC	Immunohistochemistry
BRCA	Breast cancer (gene)	LCIS	Lobular carcinoma in situ
CAIX	Carbonic anhydrase IX	LDHA	Lactate dehydrogenase A
CAF	Cancer-associated fibroblast	LH	Luteinizing hormone
CBP/p300	CREB binding protein	LHRH	LH-releasing hormone
CCND1	Cyclin D1 (gene)	LOH	Loss of heterozygosity
CDH1	E-cadherin (gene)	MAPK	Mitogen activated protein kinase
CGH	Comparative genomic hybridization	MMP	Matrix metalloproteinase
CHK1	Checkpoint kinase 1 (gene)	MSC	Mesenchymal stem cell
CIS	Carcinoma in situ	N-CoR	Nuclear receptor co-repressor
CK	Cytokeratin	NHG	Nottingham histological grade
CREB	cAMP responsive element binding protein	PAK1	p21 activated kinase 1 (gene)
CTTN	Cortactin (gene)	PHD	Prolyl hydroxylase
DBD	DNA-binding domain	PI3K	Phosphatidylinositol 3-kinase
DCIS	Ductal carcinoma in situ	PKA	Protein kinase A
DNA	Deoxyribonucleic acid	PR	Progesterone receptor
E2	Estradiol	RB	Retinoblastoma (gene)
ECM	Extracellular matrix	RFS	Recurrence-free survival
EGFR	Epidermal growth factor receptor	RTK	Receptor tyrosine kinase
EMT	Epithelial-mesenchymal transition	SERD	Selective estrogen receptor downregulator
ER	Estrogen receptor	SERM	Selective ER modulator
ERE	Estrogen response element	SN	Sentinel node
ERK	Extracellular signal regulated kinase	SRC	Steroid receptor co-activator
FADD	Fas-associated death domain	TAM	Tumor-associated macrophage
FAK	Focal adhesion kinase	TCA	Tricarboxylic acid
FGFR	Fibroblast growth factor receptor	TDLU	Terminal duct lobulo-alveolar unit
FIH-1	Factor inhibiting HIF-1	TGF- β	Transforming growth factor- β
FSH	Follicle-stimulating hormone	TMA	Tissue microarray
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	VEGF	Vascular endothelial growth factor
GPCR	G-protein-coupled receptor	VHL	Von Hippel-Lindau
GRK	GPCR kinase		

Introduction to Cancer

Malignant Progression

The transformation of normal cells to malignant cancer cells is a complex multistep process, characterized by an accumulation of various genetic alterations. Malignant progression is thought to involve certain steps allowing the cancer cells to overcome specific cellular mechanisms that would normally prevent them from growing. These growth advantages, due to genetic errors, are crucial for the cancer cell to develop and continue to proliferate^{1,2}. Specific traits that denote cancer cells include; the ability to divide despite growth-inhibitory signals, responsiveness to self-supplied growth factors, resistance to apoptotic signaling and a limitless potential of replication. In addition, malignant cells have the unique capability of providing for their own blood supply through induction of angiogenesis, as well as invading the surrounding tissue and entering the circulation in order to metastasize². Genomic instability is a fundamental hallmark of cancer cells and is associated with activation (gain of function) of oncogenes and inactivation (loss of function) of tumor suppressor genes. Oncogenes are cancer-promoting genes and include genes that when overexpressed or expressed as a mutated variant promote tumor cell survival, often as a result of constitutive activation or overexpression of growth factor receptors and signaling components. In contrast, tumor suppressor genes are growth-inhibitory and when inactivated subsequently promote uncontrolled cell growth³. Both alleles of a tumor suppressor gene have to be lost for a non-functional protein product, whereas oncogene activation occurs if a single allele is genetically altered⁴. Besides mutations and chromosomal rearrangements, inactivation of tumor suppressor genes can occur via DNA hypermethylation of the gene promoter, and this phenomenon is referred to as epigenetic silencing⁵. Two additional hallmarks of cancer have recently been proposed; evasion of anoikis – cell death signals mediated by loss of cell-extracellular matrix (ECM) contact, and resistance to local acidification toxicity caused by elevated glucose consumption⁶.

The Origin of Cancer

Tumor progression is described as a successive accumulation of beneficial genetic alterations leading to clonal expansion of cells. All genetic events required for tumorigenesis do not occur in a single cell, they are rather acquired over time in the

subpopulation of cells originating from the tumor-initiating cell, gradually resulting in an altered phenotype. The evolution of cancer cell populations has been debated in the past decade. Two theories explaining the origin of cancer have been proposed, and these concepts have few similarities. The first, the cancer stem cell hypothesis, which has received a great deal of attention recently, states that only a specific subset of tumor cells are capable of driving tumorigenesis. These cells are referred to as cancer stem cells and are able to self-renew indefinitely as well as differentiate. Tumor heterogeneity is according to this model achieved by the potential of these stem cells to produce any cell type of a tumor. The cancer stem cell is thought to mainly originate from a normal stem or progenitor cell and to constitute only a small fraction of cells in a tumor⁷⁻⁹. These cells are long-lived and the mutations required to become a tumor cell can accumulate more easily. The last couple of years much evidence supporting this hypothesis has been generated^{10, 11}. The observed requirement of transplantation of a large number of cells to acquire tumor formation substantiates this model¹². Furthermore, the cancer stem cells have been suggested to be the source of disease recurrence and treatment failure, two major clinical challenges of cancer therapy today¹¹.

The second, more well-established hypothesis, referred to as the clonal evolution model, states that cancer originates from a random single cell that has acquired multiple mutations, rather than from a stem-like cell. Clonal expansion of this cell, due to a growth advantage, will eventually result in tumor formation. The finding that the pattern of genetic alterations in the metastatic tumor cells in most cases is identical to that of the primary tumor cells supports this model. According to the clonal evolution model the property of self-renewal is an acquired feature, not seen in the cell of origin, whereas the stem cell model suggests that the ability of self-renewal is a feature of the cell of origin. In this model heterogeneity is achieved by additional beneficial mutations in the offspring resulting in new characteristics and subpopulations^{1, 13, 14}. One hypothesis does not necessarily exclude the other, rather, it is conceivable that they both contribute to tumor progression¹⁰.

The Normal and Malignant Breast

Breast Development

Development of the mammary gland occurs during puberty, and the epithelium becomes fully matured at pregnancy and lactation in response to hormones¹⁵. The structure of the female breast is constituted by a branching network of ducts and lobules, surrounded by stromal and adipose tissue (Figure 1). The terminal ducts and lobules are composed of two layers of epithelial cells; the inner polarized luminal epithelial layer and the outer contractile myoepithelial layer¹⁶. The basement membrane separates the epithelial layers from the stroma. During puberty the ducts begin to branch and terminal end buds, the origin of the terminal duct lobulo-alveolar units (TDLUs) or lobules, begin to form. These TDLUs are composed of alveoli and are the milk-producing units of the breast that become terminally differentiated during lactation. Post lactation involution of the lobules occurs and they return to a stage that resembles that of the non-pregnant gland¹⁷.

Proliferation of the breast epithelium is to a large extent accounted for by the luminal cell population. The distinct expression pattern of cytokeratins (CKs) distinguishes the luminal (CK 8, 18 and 19) from the myoepithelial (CK 5, 14 and 17) cells^{18,19}. Another characteristic of the luminal epithelial cells is the expression of the estrogen receptor (ER) and the progesterone receptor (PR). A distinct subset of these cells (10-20 %) express the two steroid receptors, and these receptors have been shown to co-localize on homogeneously distributed cells of the lobules. Only a low fraction of cells proliferate and these cells do not exhibit steroid receptor expression but are rather adjacent to the receptor expressing cells, that stimulate proliferation in a paracrine fashion^{20,21}. In contrast to normal breast tissue, more than 70 % of invasive ductal carcinoma cells express ER, and in this setting these cells are proliferating in response to estrogen²². Both the luminal and the myoepithelial cells are believed to be derived from a common breast epithelial precursor cell²³. Breast epithelial stem cells are thought to account for continuous cell renewal during the different stages of breast development. The theory of the adult mammary stem cell was founded in the late 1950s when DeOme and co-workers observed that an entire mammary ductal tree could be reconstituted from epithelial tissue of different regions of the gland when transplanted²⁴.

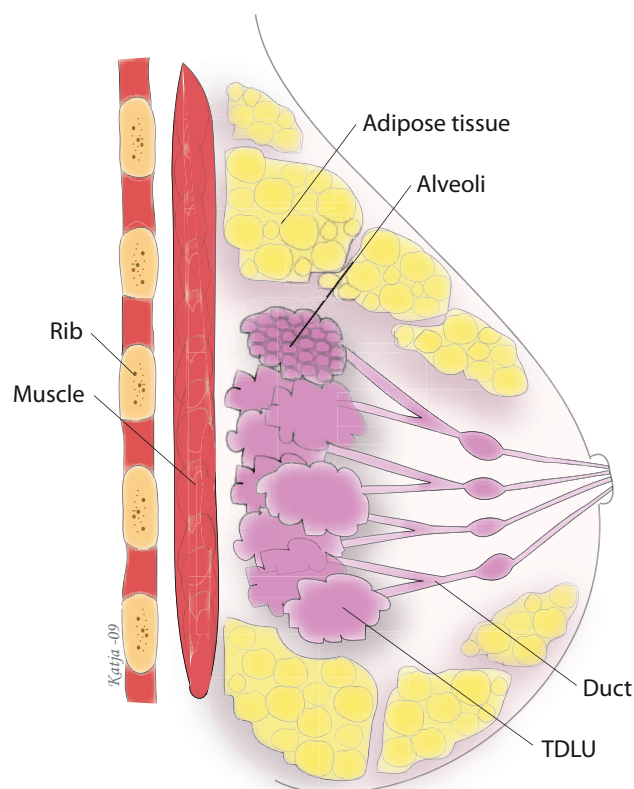


Figure 1. Schematic illustration of the breast. The normal breast consists of the functional units; the ducts and lobules (TDLUs) surrounded by adipose and stromal tissue. The lobules are composed of alveoli, the milk-producing units.

Breast Cancer

Epidemiology and Etiology

Breast cancer is the most commonly occurring malignancy in women, with more than one million new cases diagnosed each year, and is one of the leading causes of cancer-related deaths in women worldwide²⁵. The lifetime risk of developing breast cancer among Swedish women is 10 %, and the annual incidence approximately 7000 cases. The 5-year breast cancer survival rate is 87 % and the 10-year survival nearly 80 % (The national Board of Health and Welfare, 2009). Breast cancer incidence rates have been increasing over the past two decades, while mortality has decreased. Approximately 5-10 % of breast cancers are the result of genetic predisposition, while the remaining majority represents the sporadic cases. Known risk factors affecting the susceptibility to breast cancer, apart from age and heredity are hormone-related factors such as long-term exposure to hormones and the hormonal milieu. Hormonal risk factors are; early menarche, late menopause, nulliparity, and late age at first full-term pregnancy, as well as exogenous hormones such as oral contraceptives and hormone replacement therapy²⁶⁻²⁸. Lifestyle factors such as socio-economic status, increased fat-intake, high alcohol consumption and physical inactivity are also potential risk factors^{29, 30}. One of the major contributing factors to a reduced mortality is mammographic screening, introduced over 30 years ago, which involves routinely screening of women between

50 and 69 years of age³¹. Accordingly, breast cancers can be detected at an earlier stage and hence, disease progression may be prevented. In addition, implementation of improved treatment protocols, with more effective therapeutics and development of individualized therapy has been pivotal for breast cancer therapy in recent years.

The Biology of Breast Cancer

The majority of breast cancers originate from a relatively short segment of the luminal epithelia of TDLUs, and this region may be a niche where normal stem cells reside^{32, 33}. The progression from normal breast tissue to invasive cancer generally follows a specific pattern starting with benign proliferative changes, with subsequent evolution to atypical hyperplasia, carcinoma *in situ* (CIS), invasive carcinoma and finally progression into metastatic disease (Figure 2)^{34, 35}. Carcinoma *in situ* is a premalignant lesion of the mammary ducts or lobules (DCIS and LCIS respectively). The most common form of CIS is DCIS and during early stages of the disease there is hyperproliferation of cells that accumulate in the duct lumen, but without disruption of the basement membrane. DCIS is a precursor to invasive ductal carcinoma (Figure 3) and accounts for almost 20 % of all breast carcinomas detected by screening mammography^{35, 36}. However, at diagnosis approximately 15 % of DCIS patients are also presented with invasive breast cancer^{37, 38}. Invasive breast cancers are believed to mainly originate from *in situ* carcinomas, and genomic alterations accumulating during breast cancer progression can be traced back to the CIS. The major diagnostic criterion distinguishing an invasive cancer from a CIS is the absence of an intact myoepithelial cell layer. The myoepithelial cells are believed to have a pivotal role in maintaining tissue polarity and are rarely transformed, but may instead be acting as natural tumor suppressor cells^{39, 40}.

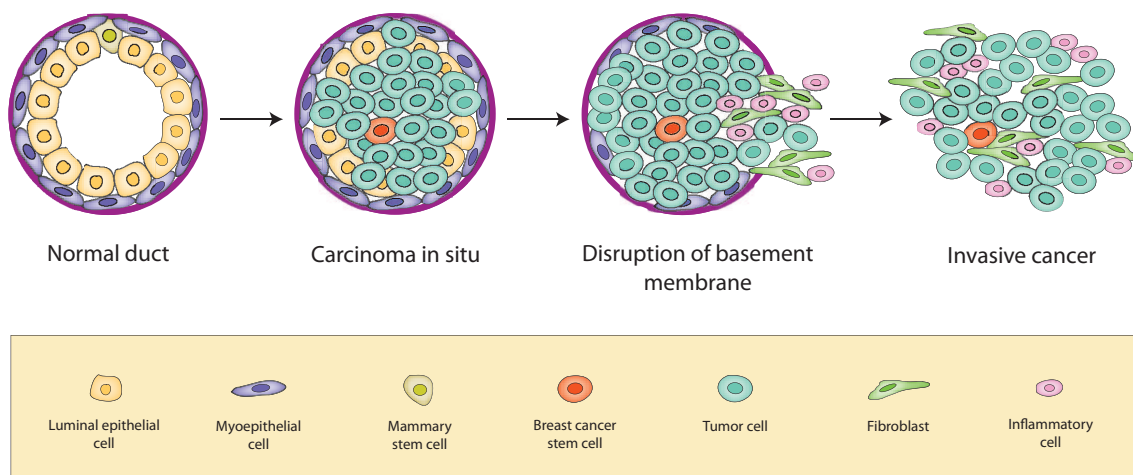


Figure 2. The progression of breast cancer from a normal duct to invasive ductal carcinoma. In the invasive cancer myoepithelial cells are not present, whereas the abundance of stromal cells such as fibroblasts and immune cells play an important role in tumor progression.

Breast cancer is a highly heterogeneous disease both clinically and at the molecular level, making it differ significantly in both prognosis and treatment response between patients. Five major subtypes of breast cancer, revealed by comprehensive gene expression profiling of large sets of tumors by multiple independent groups and technologies have been identified; luminal A, luminal B, HER2⁺/ER⁻, basal-like and normal breast-like⁴¹⁻⁴³. The different subtypes are evident already at the preinvasive stage and are distinct from the well established subtypes based on histological appearance, described below⁴⁴. The basal-like subtype, which is mainly ER, PR and HER2-negative (triple-negative) is associated with the worst clinical outcome, and is more prevalent in patients with hereditary disease. The ER⁺ luminal A-type has the best prognosis and is more commonly diagnosed in postmenopausal women⁴⁵.

The presence of a putative breast cancer stem cell, identified by the expression profile CD44⁺/CD24^{-/low} has been described^{46, 47}. Interestingly, a high fraction of CD44⁺/CD24^{-/low} cells in a breast tumor may be linked to presence of distant metastasis, however, prevalence of these cells has not been reported to be associated with reduced patient survival⁴⁸.

As previously described, tumor progression is strictly dependent on genetic alterations, and in breast cancer a vast number of different changes, that favors unrestrained growth, have been identified. Inactivating mutations or loss of the two tumor suppressor genes *BRCA1* and *BRCA2* are specifically linked to hereditary breast cancer, whereas other forms of cancer are associated with a distinct set of specific genetic alterations. Certain genetic alterations such as inactivating mutations of *TP53* or loss of *RB*, as well as oncogenic activation of *MYC* or *CCND1* are commonly observed in a variety of different cancers^{4, 49-51}.

Prognostics

Tumor Type

The specific subtype of invasive breast cancer is determined at diagnosis and is based on microscopic appearance and growth pattern. The largest subgroup, comprising approximately 75 % of breast cancers, is the invasive ductal carcinoma, while lobular carcinoma represents around 15 %. Some of the smaller remaining subtypes; tubular, medullary, mucinous, papillary, inflammatory and metaplastic breast cancer are each accountable for a very small fraction (1-2 %) ⁵². The histopathological characteristics differ significantly between subtypes and are associated with varying prognostic impact. Moreover, different histological subtypes have been shown to have slightly distinct patterns of genetic alterations².

Histological Grade

The routinely used Nottingham Histological Grade (NHG) classification is an assessment of three different morphological features; the degree of tubule formation, nuclear pleomorphism and mitotic count, which represents a collective description of differentiation grade. Each parameter is scored as 1, 2 or 3 and the total score sum defines the aggressiveness of the tumor. NHG index I (3-5) defines a well differentiated tumor, NHG II (6-7) a moderately differentiated, and NHG III (8-9) a poorly differentiated tumor ⁵³.

Tumor Stage

By the use of TNM classification (0-IV), further clinical information about the extent and progression of a specific cancer can be obtained. The three parameters primary tumor size (T), lymph node status (N) and metastatic spread (M) are taken into account. Tumor size is based on measurements of the invasive component, where a larger lesion is associated with a worse prognosis. Nodal stage is defined as the number of lymph nodes engaged, where stage I includes patients presented with invasive cancer showing no nodal involvement, and stage III involvement of four or more nodes. A metastatic tumor is defined as a stage IV cancer (Swedish Breast Cancer Group, 2009).

Novel Prognostic Criteria

In order to predict the clinical course of a specific tumor and to be able to individualize breast cancer therapy, gene expression profiling of individual patients can in addition be employed. To date various prognostic tests are available, including the Rotterdam 76-gene set, Invasive Gene Signature, *Oncotype DX*TM and *MammaPrint*[®] ⁵⁴.

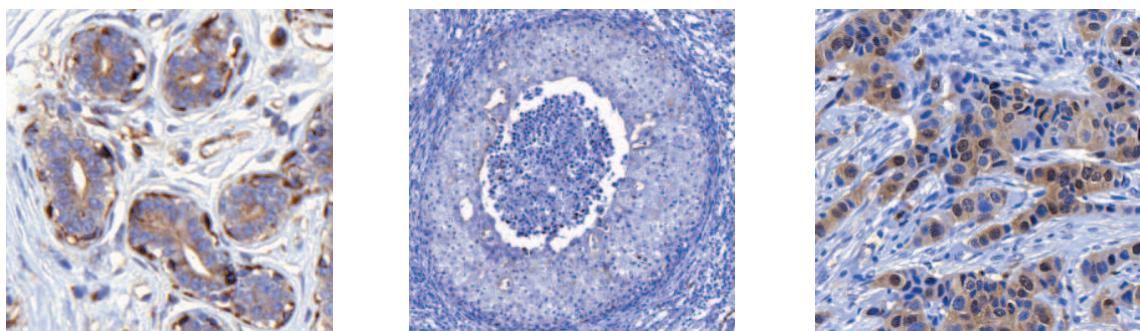


Figure 3. Immunohistochemical stainings of tissue from different stages of breast cancer development. Invasive ductal carcinoma originates from the normal ducts (left panel). DCIS are often presented with a central necrosis due to poor blood supply of rapidly proliferating cells that accumulate in the duct lumen (middle panel). In the invasive lesion the ductal structures are completely disrupted (right panel).

Breast Cancer Therapy

Surgery

Conventional breast cancer therapy includes removal of the tumor either by mastectomy (removal of the whole breast) or by breast conserving surgery, depending on the extent of the tumor. The treatment option of breast conserving surgery has become the leading treatment of choice, but is indispensable for patients with large tumors or multifocal disease. Since introducing the sentinel node (SN) biopsy, where only the nodes likely to be involved are removed, the number of side effects associated with surgery has been greatly reduced. The SN technique is based on removal and examination of the first axillary lymph node that drains the breast, and if no malignant cells are encountered here it is unlikely that the cancer has spread ⁵⁵.

Radiotherapy

Surgery in combination with local irradiation of the breast will cure the majority of breast cancer patients. The main aim of radiotherapy is to reduce the risk of local recurrence, and it has been shown to significantly improve patient survival ^{56, 57}. Radiotherapy is the standard postoperative treatment for patients receiving breast conserving surgery and is given locally to avoid damage to surrounding tissue.

Chemotherapy

In order to minimize the chances of continued growth of potential residual cells, adjuvant systemic therapy such as chemotherapy or endocrine therapy is administered to breast cancer patients in most cases. Chemotherapy is the treatment of choice for hormone receptor negative patients and is mainly given as a combination of several different agents, referred to as polychemotherapy, potentially providing a synergistic effect. It is given both in the neo-adjuvant and the adjuvant settings, as well as palliatively. The most commonly used polychemotherapies are CMF (cyclophosphamide, methotrexate, 5-fluorouracil), FEC (5-fluorouracil, epirubicin, cyclophosphamide) and FAC (5-fluorouracil, doxorubicin, cyclophosphamide) ⁵⁸. Additionally, the taxanes docetaxel and paclitaxel are applicable for second-line therapy of advanced disease ⁵⁹. Well-known disadvantages with these agents are resistance and severe toxicity to normal tissue ⁶⁰.

Endocrine Therapy

Standard endocrine therapy for breast cancer patients is based on inhibiting estrogen action, either by blocking ER or by inhibiting estrogen synthesis. At least 70 % of breast cancers express the ER, and since the tumor is dependent on estrogen for maintained growth, interference with the different steps of the estrogen pathway greatly reduces tumor growth. Consequently, ER serves as a clinically useful predictive marker in guidance to treatment of hormone receptor-positive disease ⁶¹. The progesterone receptor is a target of ER transcription and its presence in breast cancer is an indirect marker for functional ER signaling. PR has emerged as an even stronger predictor for endocrine therapy-response than ER, speculatively explaining how some patients showing ER-negative tumors still respond to endocrine therapy ⁶²⁻⁶⁴. The choice of endocrine therapy is mainly based on the menopausal status of the patient. In premenopausal women complete suppression of estrogen synthesis is associated with negative side-effects due to a disrupted systemic hormone balance.

SERMs and SERDs

The selective ER modulator (SERM) tamoxifen (Nolvadex) has been the first-line adjuvant treatment option for ER-positive breast cancers of all stages the past 30 years ⁶⁵. Results from prevention trials have shown that tamoxifen lowers breast cancer incidence by 30-40 % in high-risk patients ⁶⁶. Tamoxifen inhibits the growth-stimulatory effect of estrogen, mediated by competitive binding to ER, and additionally favors recruitment of co-repressors rather than the co-activator recruitment that occurs when estrogen binds to ER. Binding of tamoxifen also results in a receptor conformation distinct from the one caused by estrogen-binding, however dimerization and DNA-binding can still occur ⁶⁷. While acting as an antagonist in breast, tamoxifen has an agonistic effect in bone, uterus and the cardiovascular system ^{68, 69}.

Raloxifene (Evista), another SERM was developed as a treatment for patients with tamoxifen refractory breast cancers ⁷⁰. The efficacy of raloxifene is similar to that of tamoxifen, but it has been suggested to be a safer SERM due to reduced negative effects in endometrial cells ⁷¹.

Pure anti-estrogens, including the selective ER downregulators (SERDs) have been introduced with the anticipation to overcome the non-beneficial agonistic effects of the SERMs. The steroidal anti-estrogen fulvestrant (Faslodex) is a SERD that binds ER and inhibits dimerization and DNA-binding. It antagonizes both the AF-1 and the AF-2 domains of the ER (detailed below), whereas SERMs only inhibit AF-2, and cause complete suppression of estrogen-dependent gene transcription. Data from clinical trials comparing the efficacy of fulvestrant and tamoxifen show a more efficient blockade of the ER pathway for fulvestrant ^{72, 73}.

Aromatase Inhibitors

An alternative strategy of estrogen deprivation is the use of aromatase inhibitors (AIs), which interfere with the synthesis of estrogens. AIs inhibit the enzyme aromatase, the ultimate source of all steroidal estrogens, which converts androgens to estrogens. These agents are the primary treatment of choice for postmenopausal breast cancer patients, with early and advanced ER-positive disease. The third-generation AIs anastrozole (Arimidex) and letrozole (Femara) are non-steroidal compounds reversibly inhibiting aromatase⁷⁴. The efficacy of anastrozole has been reported to be higher compared to tamoxifen and it is also better tolerated, in postmenopausal breast cancer patients^{75, 76}.

HER2 Targeted Therapy

Overexpression of the human epidermal growth factor receptor2 (HER2), often due to amplification of its corresponding gene, is observed in 20-30 % of breast cancers⁷⁷. HER2 serves both as a prognostic and a treatment predictive marker in breast cancer. The monoclonal antibody trastuzumab (Herceptin), that prevents receptor dimerization and hence blocks signaling, is the first-line treatment option for patients displaying HER2-positive breast cancers. It has recently been recognized that a combination of anti-estrogens and Herceptin may improve survival in hormone receptor-, HER2-positive breast cancer patients, and also reduce the risk of endocrine resistance associated with HER2 overexpression. Alternative anti-HER2 therapeutics include the tyrosine kinase inhibitors lapatinib and gefitinib that block intracellular signaling through HER2 and EGFR⁷⁸.

Estrogen Receptor Signaling and Endocrine Resistance

Estrogen

Mammary gland development during puberty and onward is governed by hormone signaling, mainly mediated by estrogen and progesterone. Estrogen is a steroid hormone primarily synthesized by the ovaries. Its synthesis is regulated by luteinizing hormone (LH)-releasing hormone (LHRH) released from the pituitary gland. LHRH regulates release of LH and follicle-stimulating hormone (FSH), which in concert increase intracellular levels of cAMP in the ovary, activating cAMP-response-element-binding protein (CREB). This ultimately results in elevated levels of aromatase, the enzyme that catalyses the conversion of ovarian androgens to estrogens⁶¹. The predominant estrogen in premenopausal women is 17 β -estradiol (E2), whereas estrone is the main estrogen in postmenopausal women. Estrogen regulates a number of biological processes, including proliferation, development and tissue-specific gene regulation in the reproductive tract, central nervous system and bone⁷⁹. After menopause the production of estrogen from the ovaries ceases, but estrogen is still produced at other sites, such as subcutaneous fat, breast and bone⁸⁰. Tissue types that are regulated by estrogen signaling can be divided into two groups; the first or classical in which ER α is expressed at high levels and the second or non-classical where ER α expression is low or even undetectable (detailed below)²². While playing a critical role in development of the mammary gland and acting as protective mediator against osteoporosis and cardiac disorders, estrogen also promotes development of hormone-dependent breast cancers⁸¹.

Estrogen Receptors

The estrogen receptors exist in two forms, the ER α and the ER β , which are products of two distinct genes located on different chromosomes. The ERs belong to the steroid/thyroid/retinoid hormone superfamily of nuclear receptors and have distinct tissue- and cell-type specific expression patterns. The sequence homology of the DNA-binding domain (DBD) is conserved both structurally and functionally between the two receptors. The N-terminal regions however, differ between ER α and ER β , a proposed explanation for the diversity of responsiveness to different ligands. The ERs contain a constitutively active transcription-activating function; AF-1, located in the N-terminal, and a second estrogen-inducible AF-2 domain located

C-terminally (within the ligand-binding domain). The DBD is located in between the AF-1 and AF-2 domains. The activity of the AFs contributes to estrogen-mediated transcription of ER target genes. Binding of estrogen induces a conformational change of the receptor, followed by dimerization and DNA-binding, resulting in recruitment of co-regulators and other transcription factors, ultimately forming the preinitiation complex required for transcriptional activation ⁷⁹. ER-mediated transcription occurs at estrogen response elements (EREs) of target genes, and both ERs have similar affinity for endogenous estrogen and similar specificity and affinity for ERE binding ⁸². Approximately 70 % of ER target genes are downregulated following estrogen stimulation and many of these genes are transcriptional repressors or associated with growth arrest and apoptosis. ER gene transcription can be regulated in different ways, including direct DNA-binding as homo- or heterodimers (classical pathway), or through interaction with other transcription factors, such as activating protein-1 (AP-1) (non-classical pathway).

Expression of ER α is found specifically in the uterus, liver, kidney and heart, whereas ER β expression is confined to ovary, prostate, lung, gastrointestinal tract and bladder, as well as hematopoietic and central nervous systems. Co-expression of the receptors is observed in mammary gland, epididymis, thyroid gland, adrenal glands, bone and certain regions of the brain ⁷⁹. In the breast, ER α is expressed both in ductal epithelium and stroma, but not in lobular epithelium, whereas expression of ER β can be detected in all three compartments. Knowledge on ER β biology and functionality is limited, and if not specified ER will refer to the ER α in the following paragraphs. The spectrum of ligands vary slightly between ER α and ER β , with some ligands exhibiting preferential binding to one or the other ⁸³. Moreover, the action of ER α and ER β can be opposing depending on the tissue type, cell type or the nature of the ligand ⁸⁴. Apart from the extensively studied nuclear ER, presence of cytoplasmic and membrane-associated ERs have also been described ^{85, 86}. These extra-nuclear receptors are thought to be derived from the same transcript as the nuclear, but are exported from the nucleus. The membrane-localized ERs signal through kinase cascades as well as other second messengers to induce transcriptional regulation ⁸⁷.

Tamoxifen Resistance

Despite the presence of ER, approximately 50 % of breast cancers develop endocrine resistance, a major clinical limitation in breast cancer therapy^{88, 89}. The mechanisms behind this phenomenon are being extensively studied, and imply a complex signaling network governing ER function and interaction with various co-regulators⁹⁰⁻⁹³. Tamoxifen resistance in particular, has been described to be dependent on a vast number of different molecular mechanisms. However, information regarding resistance mechanisms associated with SERDs and AIs is still insufficient. The main predictor of endocrine therapy response is expression of ER, and lack of ER expression is the major mechanism of *de novo* or intrinsic resistance. However, the majority of resistant breast cancers retain expression of ER, and are hence still responsive to a second-line endocrine therapy such as the pure anti-estrogens or AIs^{94, 95}. However, only 20 % of tamoxifen resistant patients eventually respond to fulvestrant or AIs^{96, 97}. The opposing effects of tamoxifen observed due to tissue-specificity seem to be dependent on regulation of the AF-1 and AF-2 domains of the ER. Tamoxifen-binding to ER prevents activation through AF-2, but AF-1 mediated transcription can still occur, and this may explain the partial agonism observed for this SERM⁹⁸. In breast tissue ER transcriptional activity is mainly dependent on AF-2 activation and the effect of tamoxifen is antagonistic, whereas in bone and uterus the activity is mainly AF-1 dependent, resulting in an agonistic action of the drug.

Mechanisms Underlying Tamoxifen Resistance

As previously noted, a large number of potential mechanisms have been suggested to play a role in acquired resistance to endocrine therapy (Figure 4). The milieu of co-regulators seems to be an essential player in the agonist/antagonist profile of tamoxifen⁹⁹. Several different co-factors associated with ER have been suggested to confer tamoxifen resistance. Phosphorylation of co-activators such as amplified in breast cancer 1 (AIB1), also known as steroid receptor co-activator-3 (SRC-3), enhances the interaction and/or activation of ER, whereas phosphorylation of co-repressor such as nuclear receptor co-repressor (N-CoR) inhibits these interactions¹⁰⁰. AIB1 overexpression, as well as low N-CoR expression has been linked to tamoxifen insensitivity in breast cancer^{101, 102}. Moreover, high expression of cyclin D1 has been reported to confer tamoxifen resistance^{103, 104}. Overexpression of cyclin D1 promotes recruitment of SRCs causing estrogen-independent transactivation of ER, resulting in activation of ER target genes^{105, 106}. Treatment with tamoxifen *in vitro* has been reported to redistribute cyclin D1 binding from a cyclin D1-STAT3 complex to the ER α , resulting in activation of both STAT3 and ER α , supporting the crucial role of cyclin D1 in endocrine resistance¹⁰⁷.

Another mechanism believed to be involved in tamoxifen resistance is cross-talk between ER and growth factor receptor signaling pathways. ER can be activated by

phosphorylation via EGFR, PI3K/AKT and HER2 signaling in a ligand-independent fashion, causing transcriptional activation of ER target genes¹⁰⁸. Consequently, overexpression of growth factors or constitutively active receptors, often observed in cancer, may render tumor cells resistant to anti-estrogen therapy. ERK has been reported to phosphorylate ER and activate target gene transcription⁹¹. Furthermore, overexpression of HER2 has been shown to be associated with an impaired tamoxifen response in ER-positive breast cancer *in vitro*¹⁰⁹. HER2 inhibition with Herceptin, as well as inhibition of MAPK was reported to restore tamoxifen sensitivity in resistant human breast cancer cells expressing high levels of HER2¹¹⁰. HER2 overexpression has been described as one of the most well characterized mechanism of endocrine resistance¹¹¹. Notably, several reports have failed to substantiate this well-established concept¹¹².

A considerable number of studies have reported tamoxifen resistance to be dependent on specific markers exhibiting an altered expression in breast cancer. For example, loss of PR has been linked to impaired response to tamoxifen^{63, 113}. Other markers suggested to be involved in clinical insensitivity to tamoxifen are Pak1 and PKA^{93, 114}. A specific phosphorylation of ER at serine 305 (ER α S305-P) by PKA is linked to an agonistic effect of tamoxifen, due to a conformational change of ER and recruitment of the co-activator SRC-1 *in vitro*¹¹⁶. Furthermore, ER α S305-P has been associated with resistance to adjuvant tamoxifen in premenopausal breast cancer patients¹¹⁷.

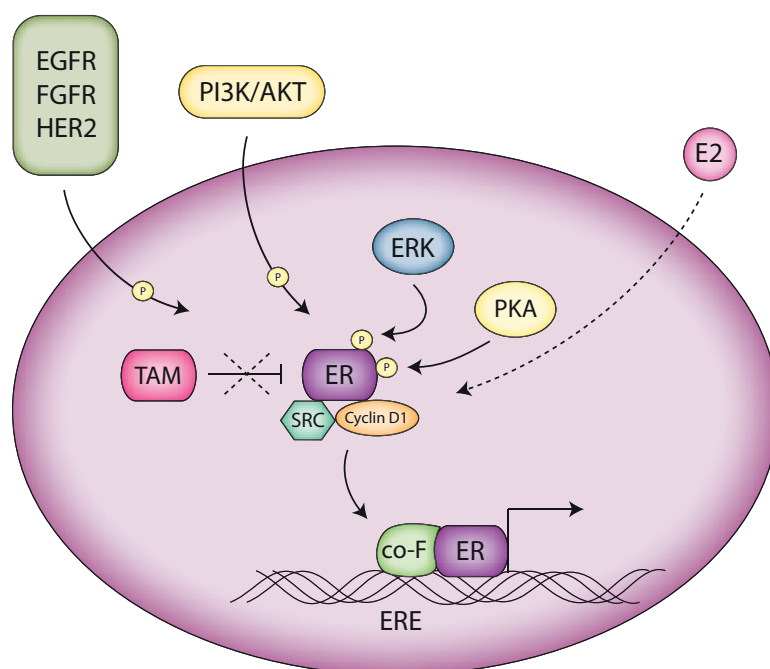


Figure 4. Schematic representation of mechanisms contributing to tamoxifen resistance. Phosphorylation of ER by components of many different signaling pathways can activate ER in a ligand-independent fashion, abolishing the effect of tamoxifen (TAM). SRC and Cyclin D1 act as co-activators of ER target gene transcription.

Additionally, involvement of ER β in tamoxifen resistance has been reported, implying a beneficial effect on tamoxifen response in patients exhibiting ER β -positive tumors^{118, 119}. ER itself can also activate other transcription factors and enhance transcription of target genes involved in growth factor signaling pathways¹²⁰. Moreover, the specific ligand, receptor subtype, receptor phosphorylation and promoter sequence of a certain target gene are all crucial for the balance of a beneficial effect of endocrine agents. Interestingly, mutations in ER are rarely found and are not likely to contribute to endocrine resistance. However, a truncated variant of ER α (ER α 36) has been reported to be linked to reduced responsiveness¹²¹, as well as a mutation resulting in a hypersensitive ER, that by enhanced binding of co-activators can be activated even at very low estrogen levels¹²².

In recent years genome-wide gene expression analyses of clinical material have been employed in the search for potential mechanisms of endocrine resistance, and a number of distinct gene signatures predictive for tamoxifen sensitivity in breast cancer patients have been identified. Several biological processes such as proliferation, apoptosis, invasion and cell motility have been implicated in responsiveness, and a number of specific signaling pathways have emerged as particularly important¹²³. By using this approach for identification of biomarkers a large number of genes are found, but the knowledge about their specific mechanisms in conferring resistance is limited. Future analyses considering the complete biological system of cellular responses and pathways are warranted to gain a better understanding of the intricate mechanisms underlying endocrine resistance.

It is conceivable that different mechanisms are required to confer resistance depending on the specific anti-estrogen administered. Moreover, changes in the conformation of ER as well as specific phosphorylation patterns seem to play a crucial role for anti-estrogen insensitivity. Clinical implications to circumvent endocrine resistance may be to target different co-regulators or signaling pathways involved in ER regulation. The search for predictive markers is constantly expanding, and the main aim is to identify novel strong predictors for tamoxifen response, in order to improve and individualize breast cancer therapy.

Genomic Aberrations at Chromosome 11q

Amplifications and Deletions

Gene amplification is a genetic alteration frequently observed in various cancers and a number of specific chromosomal regions are known to be common targets of this event. Amplification of genes such as *HER2* (17q12), *CCND1* (11q13) and *MYC* (8q24) are found in several different cancers and have been associated with poor prognosis¹²⁴⁻¹²⁶. The discovery of potential candidate genes responsible for the emergence and maintenance of specific amplified regions has been important in furthering our understanding of carcinogenesis. In addition, deletion of certain chromosomal regions is also commonly described in cancer. Loss of heterozygosity (LOH) at 3p, 7q, 10q, 11q, 13q, 17 and 22q are aberrations frequently involved in breast cancer¹²⁷. Notably, amplified regions are presumed to include oncogenes, whereas deleted regions include tumor suppressor genes⁵⁰.

Chromosome 11q: Genetic Events and Gene Products

Numerous studies describe amplification of 11q13 in breast cancer and its association with reduced patient survival¹²⁸⁻¹³¹. One of the most extensively studied genes included in this chromosomal region is *CCND1*, and this gene in concert with a number of others, such as *FGF3*, *FGF4*, *PAK1*, *FADD* and *CTTN* (cortactin) have been proposed as putative driver genes of this specific amplification. Within the 11q13 locus four different amplicons have been identified, and these can be amplified concurrently as well as independently of each other^{50, 132-134}. The function and implication in tumor progression denoting some of the gene products involved in the 11q13 amplification event will be outlined below.

Cyclin D1

The *CCND1* gene encodes the cell cycle regulating protein cyclin D1, essential for the progression through G1- into S-phase of the cell cycle. Following complex formation between cyclin D1 and CDK4/6, phosphorylation of the retinoblastoma protein (pRb) occurs and the cell enters the cell cycle¹³⁵. Expression of cyclin D1 is crucial for lobulo-alveoli formation during normal mammary gland development^{136,137}, and deregulation of the protein through overexpression in transgenic mice results in aberrant mammary

development and promotes tumorigenesis¹³⁸. Cyclin D1 is one of the most frequently overexpressed oncogenes in primary breast cancer⁵⁰, with elevated expression in approximately 50 % of cases^{139, 140}. Several studies report cyclin D1 overexpression to be a negative predictor of prognosis^{141, 142}, whereas others show an association to an ER-positive phenotype and improved clinical outcome^{103, 140, 143, 144}. In about 15 % of breast cancers overexpression of the protein is due to amplification of its corresponding gene *CCND1*¹⁴⁵⁻¹⁴⁷, and as previously noted, this specific amplification has been linked to poor prognosis^{131, 148}. Interestingly, overexpression of cyclin D1 has been associated with tamoxifen resistance in breast cancer, and amplification of *CCND1* has furthermore been linked to a potential agonistic effect of tamoxifen in premenopausal breast cancer patients, independently of protein expression levels^{103, 104}. Based on these evidence, cyclin D1 might serve as a potential treatment predictive marker in breast cancer therapy¹⁴⁹.

Cortactin

The cortactin gene *CTTN* (also *EMS1*) has been identified as a potential candidate gene responsible for the emergence and maintenance of 11q13 amplification in breast cancer⁵⁰, and is frequently co-amplified with *CCND1*¹⁵⁰. Cortactin is an actin-associated scaffolding protein involved in assembly and structure of actin networks¹⁵¹. It co-localizes with cadherin and β -catenin in adherens junctions, where it has a central role in intracellular adhesion¹⁵². Cortactin has been observed to localize to lamellipodia at the leading edge of the cell¹⁵³, and overexpression of the protein has been reported to promote cell motility¹⁵⁴, as well as invasion and experimental metastasis¹⁵⁵. Moreover, gene amplification and protein overexpression of cortactin is associated with poor clinical outcome in breast cancer¹⁵⁶.

FADD

The Fas-associated death domain (FADD) is an adaptor protein that interacts with the cytosolic tail of the Fas receptor, and is involved in receptor-induced apoptosis via recruitment of the initiator caspases-8 and -10 (co-factors of the death-inducing signaling complex (DISC))¹⁵⁷⁻¹⁵⁹. FADD has also been implicated in embryonic development and cell cycle control of lymphoid cells^{160, 161}. The role of FADD in tumorigenesis remains poorly understood, however, protein overexpression has been associated with poor prognosis in lung cancer¹⁶².

Loss of Distal 11q

Concurrently with amplification of 11q13, loss of heterozygosity (LOH) at the distal end of chromosome 11q has been described¹⁶³. This genetic event is closely related to the amplification of 11q13 and has been suggested as equally significant in predicting poor clinical outcome as the amplification^{164, 165}. Cell lines exhibiting loss of distal chromosome 11q exhibit defective DNA damage recognition and repair,

which might be explained by the loss of several genes critical for a functional DNA damage response pathway, such as ataxia telangiectasia mutated (*ATM*) (11q22.3) and *H2AFX* (11q23.2-11q23.3) ¹⁶⁴. These cells also show chromosomal instability further implying potentially increased cancer susceptibility and therapy resistance. Moreover, deletions at distal chromosome 11q has been described in various malignancies such as ovarian, esophageal, cervical, prostate and breast cancer, and this deletion has been associated with reduced patient survival ¹⁶⁶⁻¹⁷¹.

Chk1

CHK1, one of the potential cancer-associated genes included in the 11q deletion, encodes the protein serine/threonine kinase, checkpoint kinase 1 (Chk1), a major cell cycle checkpoint regulator ^{172, 173}. In response to DNA damage the ATM and Rad3 related (ATR) kinase phosphorylates and activates Chk1, which in turn phosphorylates and inhibits the tyrosine phosphatases Cdc25A/C, regulating inhibitory phosphorylation sites on cyclin-dependent kinases, ultimately resulting in cell cycle arrest ¹⁷⁴. Chk1 is essential for maintenance of genomic integrity and plays a fundamental role in mammalian development ^{175, 176}. High Chk1 expression has been linked to high grade triple-negative breast cancers ¹⁷⁷. Given the crucial role of Chk1 in the G1/S and G2/M checkpoints, inhibition of Chk1 has been investigated as an approach for cancer treatment, by potentiating the efficacy of chemotherapeutic agents, through abrogation of the cell cycle checkpoints ^{178, 179}. To date, a number of Chk1-inhibitors have been evaluated in clinical trials, but not yet proven to be useful anti-cancer therapeutics ¹⁸⁰.

β-arrestin1

ARRB1 is another gene that maps to the 11q13 chromosomal locus ¹⁸¹. This gene encodes the protein β-arrestin1 which belongs to a small gene family consisting of four arrestins; arrestin1 and arrestin4 (α-arrestins), and arrestin2 (β-arrestin1) and arrestin3 (β-arrestin2). Arrestin 1 and 4 are exclusively expressed in retinal rods and cones respectively, while β-arrestin1 and 2 are expressed in virtually all tissues ¹⁸². β-arrestins are versatile adaptor proteins that regulate the signaling and trafficking of G-protein-coupled receptors (GPCRs) following their activation and phosphorylation by GPCR kinases (GRKs) ¹⁸³. The binding of β-arrestin sterically prevents further G-protein signaling and hence desensitizes the receptor ¹⁸⁴. In recent years the role of β-arrestins in cell signaling has been extensively studied and their function as scaffold proteins that interact with a number of different signaling molecules has emerged ^{182, 185, 186}. Signaling pathways reported to be modulated by β-arrestins include TGF-β, IGF-1R, PI3K and MAPK pathways ^{187, 188}. Activation of the MAPK cascade induces changes in diverse cellular functions, such as differentiation, proliferation, cell motility and survival, and by acting as scaffolds for components of the MAPK signaling pathway β-arrestins may contribute to tumorigenesis ¹⁸⁹.

Single knockout mice for either β -arrestin1 or β -arrestin2 do not display grossly abnormal phenotypes, whereas a double knockout variant results in embryonic lethality¹⁹⁰. Furthermore, β -arrestin1 has been implicated in promotion of tumor growth, as well as in cell migration *in vitro* and metastatic spread to the liver *in vivo*^{188, 191, 192}. The potential involvement of *ARRB1* in 11q13 amplification has not been described, but this gene is likely to be affected by the genetic events occurring at this chromosome, and hence β -arrestin1 may play a role in the clinical outcome of various cancers.

Tumor Hypoxia

Clinical Definition of Hypoxia

Solid tumors are frequently presented with areas of reduced oxygen pressure, referred to as hypoxia. In normal, well-vascularized tissue the oxygen levels are approximately 5-6 %, whereas tumor tissue can exhibit oxygen levels below 1 %¹⁹³. Hypoxia occurs at early stages of tumorigenesis and is commonly observed in non-invasive malignant lesions¹⁹⁴. As tumors grow and expand, cells that reside more than 150 μm away from the capillaries will be subjected to an inadequate oxygen supply, thus for survival they need to adapt to these limiting conditions¹⁹⁵. A rapidly expanding tumor requires its own vasculature, but the high rate of vessel formation results in an abnormal vascular network. Consequently, regions of necrosis can often be observed in solid tumors caused by disturbed microcirculation, unable to supply the tumor cells with oxygen.

HIF-1 α Regulation

The master regulators of hypoxia are the hypoxia-inducible factors (HIFs), which are stabilized in response to low oxygen levels and subsequently induce transcription of target genes involved in the hypoxic response (Figure 5). The key regulatory factor is the heterodimeric transcription factor HIF-1 consisting of the HIF-1 α and the HIF-1 β subunits¹⁹⁶. In the presence of oxygen the HIF-1 α subunit is continuously eliminated through targeting for proteasomal degradation by the tumor suppressor protein Von Hippel-Lindau (VHL). Post-translational modification of HIF-1 α by the oxygen dependent prolyl hydroxylases (PHDs) promotes the interaction with VHL, which is part of a protein complex including a number of other proteins such as elongin-B and C, and the ubiquitin-conjugating enzyme E3¹⁹⁷. Hydroxylation by the PHDs at two proline residues of HIF-1 α , and further acetylation of a lysine residue in the oxygen-dependent degradation domain (ODDD) of the protein, is required for the association with VHL¹⁹⁸. When oxygen is absent the PHDs are inactivated and no ubiquitination and subsequent degradation of HIF-1 α will occur. This stabilization of HIF-1 α results in its translocation to the nucleus and transcriptional activation of target genes. In the nucleus HIF-1 α dimerizes with its constitutively expressed binding partner HIF-1 β or aryl hydrocarbon receptor nuclear translocator (ARNT) to form the active transcription factor HIF-1. Additional co-factors such as CBP/p300 are also recruited to the hypoxia response elements (HREs) in the enhancer and

promoter regions of target genes¹⁹⁹. HIF-1 α has two paralogs termed HIF-2 α and HIF-3 α , which are closely related to HIF-1 α . These also dimerize with HIF-1 β and regulate transcription of target genes, which for HIF-2 α partially overlap with those known to be regulated by HIF-1 α ²⁰⁰. Approximately 2 % of the human genome has been reported to be targeted by active HIF-1²⁰¹, and it is noteworthy that these genes are involved in biological processes crucial for tumor development, potentially explaining why hypoxic tumors are often more aggressive than their oxygenated counterparts. Another major mechanism controlling the activity of HIF-1 α is the factor inhibiting HIF-1 (FIH-1), which prevents the interaction of HIF-1 α with CBP/p300²⁰². FIH-1 is activated in response to an oxygen level that is lower than the one required for PHD activation²⁰³.

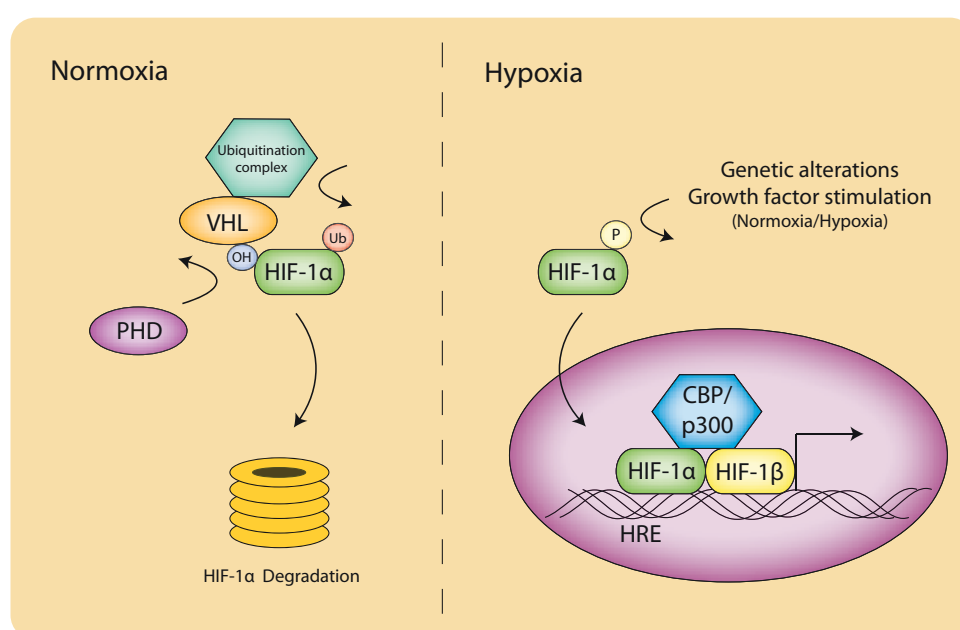


Figure 5. Schematic illustration of HIF-1 α regulation in normoxia and hypoxia. When oxygen is present HIF-1 α is ubiquitinated and degraded. In hypoxia when the PHDs are inactive HIF-1 α translocates to the nucleus where it interacts with HIF-1 β to form the active transcription factor HIF-1. Several additional mechanisms besides hypoxia, such as activation of oncogenes or growth factor stimulation, have been described to activate HIF-1 target gene transcription.

The Hypoxic Response

Hypoxia-inducible gene products facilitate adaptation to the limited oxygen supply, and are involved in numerous biological processes including proliferation, metabolism, apoptosis, chromosomal integrity, angiogenesis and migration, all of which are essential mechanisms deregulated in tumorigenesis. In order to adapt to the hypoxic conditions cancer cells take advantage of various hypoxia-induced growth-promoting signals, and cells that modify cellular mechanisms optimally for survival are selected. The hypoxic

response governed by HIF-1 is complex and the repertoire of genes affected and pathways regulated differs depending on cell type or tissue. A main requirement for cells exposed to hypoxia is reduction of energy consumption, hence hypoxic cells switch from the high energy producing, oxygen-dependent tricarboxylic acid (TCA) cycle to the more energy conserving oxygen-independent glycolysis. Various enzymes involved in the glycolytic pathway are regulated by hypoxia, including lactate dehydrogenase A (LDHA) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Other factors involved in metabolism that are affected are the glucose transporters GLUT1 and GLUT3, as well as carbonic anhydrase IX (CAIX), a commonly used marker for hypoxia^{194, 204}. Degradation of the ECM at the invasive stage of tumorigenesis is facilitated by secretion of matrix metalloproteinases (MMPs), which are also induced by hypoxia²⁰⁵. A central biological process affected by hypoxia is angiogenesis, the mechanism by which tumors are able to establish their own vasculature for supply of oxygen and nutrients. The key factor regulating angiogenesis is vascular endothelial growth factor (VEGF), and increased expression of this specific protein is a common feature of hypoxic cells. Hypoxia has also been shown to promote gene amplification and DNA breaks, and to affect DNA damage response signaling^{206, 207}.

Hypoxia in Cancer

Numerous additional mechanisms have been described to regulate the stability and activity of HIF-1 α , such as direct phosphorylation of the protein, which has been reported to occur both under normoxic and hypoxic conditions. The MAPK pathway is involved in HIF-1 α phosphorylation together with the PI3K signaling pathway^{203, 208}. Activation of oncogenes such as *HER2*, or loss of function of the tumor suppressor genes *VHL* and *PTEN*, are also potential regulatory mechanisms affecting HIF-1 α synthesis and degradation, resulting in stabilization of the protein and target gene activation, mimicking the effect of hypoxia²⁰⁹⁻²¹¹.

The mechanism underlying hypoxic regulation of tumor progression and metastasis is a key process in cancer biology and it has been extensively studied in the last two decades. Tumor hypoxia has been associated with a clinically more aggressive phenotype in various cancers²¹²⁻²¹⁵. Furthermore, by using HIF-1 α as a marker for hypoxia approximately 25-40 % of invasive breast cancers are by definition hypoxic, and a small fraction are presented with regions of around 0.1 % oxygen, referred to as severe hypoxia. HIF-1 α has been identified as an independent prognostic marker in several studies including breast cancer patients²¹⁶⁻²²⁰.

Tumor hypoxia is associated with treatment failure, hence represents a condition which requires specialized therapeutic options. There are several fundamental problems underlying therapy resistance in patients with hypoxic tumors. Presence of oxygen is essential for the effect of radiotherapy, through production of the free radicals that destroy tumor cells²²¹. Delivery of chemotherapeutic compounds to a hypoxic

tumor is also limited due to the abnormal vasculature that results in poor perfusion^{222, 223}. Moreover, hypoxic cells generally have a low proliferation rate, which further reduces the efficiency of cytotoxic drugs²²⁴. Accordingly, therapeutics directed against hypoxic tumors need to meet requirements such as high delivery efficiency and specificity, as well as selective cytotoxicity to represent good candidates. Alternative treatment strategies for hypoxic tumors, including cytotoxic compounds that are converted to active metabolites only under oxygen-limited conditions, referred to as bioreductive drugs, is an example of a novel approach to the treatment of hypoxic tumors²²⁵. Additionally, drugs that target HIF-1 α directly or targeting signaling pathways involved in the hypoxic response are also being considered as novel cancer therapeutics. Screening for potential HIF-1 inhibitors has revealed that a number of traditional anti-cancer agents are actual inhibitors of HIF-1, and these include topoisomerase I inhibitors, microtubule-targeting drugs and anthracyclines²²⁶⁻²²⁸. A clinical challenge of cancer therapy is to identify which patients would benefit from a combination of conventional therapy and HIF-1 inhibitors. The effect of this combination therapy is still uncertain and the therapeutic effect may be manifested differently between patients. A number of novel HIF-1 inhibitors have been identified and clinical trials are warranted to determine any additional potential therapeutical benefit of these compounds²²⁹.

Epithelial-Mesenchymal Transition

The Concept of EMT

Solid tumors have been described to metastasize through conversion of epithelial cells into more fibroblast-like cells, via a biological process referred to as epithelial-mesenchymal transition (EMT) ^{230, 231}. EMT is an essential cellular mechanism during embryogenesis when the body plan is created and organs start to form ²³². In the last decade the role of a pathological activation of EMT in tumorigenesis has been extensively studied and an immense amount of data supporting this hypothesis has been published. During progression to the metastatic state tumor cells are described to acquire a mesenchymal gene expression pattern as well as a spindle-shaped fibroblast-like morphology which enhances motility ²³³.

Epithelial tissue is characterized by formation of polarized layers, which are adjoined by cell-cell junctions such as tight, gap and adherens junctions, and desmosomes. Cadherins are important for stabilization of these junctional complexes, in particular E-cadherin ²³⁴. Important hallmarks of EMT are loss of polarity and cell-cell adhesion, as well as increased migratory capacity, which results from detachment from neighboring cells. Functional loss of E-cadherin occurs early during EMT, and this is a crucial event in the reorganization of tissue structure that characterizes this biological process ²³³. Other epithelial-specific genes downregulated are components of the tight junctions, including claudins and occludin, as well as the desmosomal components desmoglein (cadherin) and desmoplakin ^{235, 236}. The majority of EMT-inducing signals exert their action through modulation of transcription factors involved in either repression of epithelial-specific genes or activation of genes important for cell motility and invasiveness, resulting in induction of the EMT-programme (Figure 6) ^{233, 237}.

Regulation of EMT

Growth factors are the natural inducers of EMT during embryogenesis and since growth factor-signaling is deregulated in tumorigenesis, this can result in a pathological activation of EMT-inducing signaling pathways. Growth factors are provided by the tumor cells themselves or by surrounding stromal cells ²³³. An extensive crosstalk between different signaling pathways has been described in EMT, and many pathways have the common endpoint of E-cadherin downregulation. Central signaling

cascades in the induction of EMT include activation of RTKs such as EGFR²³⁸ and FGFR²³⁹, as well as the Wnt²⁴⁰, Notch²⁴¹ and TGF- β pathways²⁴².

Several transcription factors with a key role in EMT-induction have been identified and these include Snail²³², Slug²⁴³, Twist²⁴⁴, E12/E47²⁴⁵, δ EF1/ZEB1²⁴⁶ and SIP1/ZEB2²⁴⁷. These transcription factors share a similar basic molecular mechanism of repression, binding to conserved E-box sequences in the promoter of the E-cadherin (*CDH1*) and other epithelial-specific genes. Snail and Slug belong to the Snail superfamily of zinc-finger transcription factors that have a functional role in transcriptional repression of the E-cadherin gene²³².

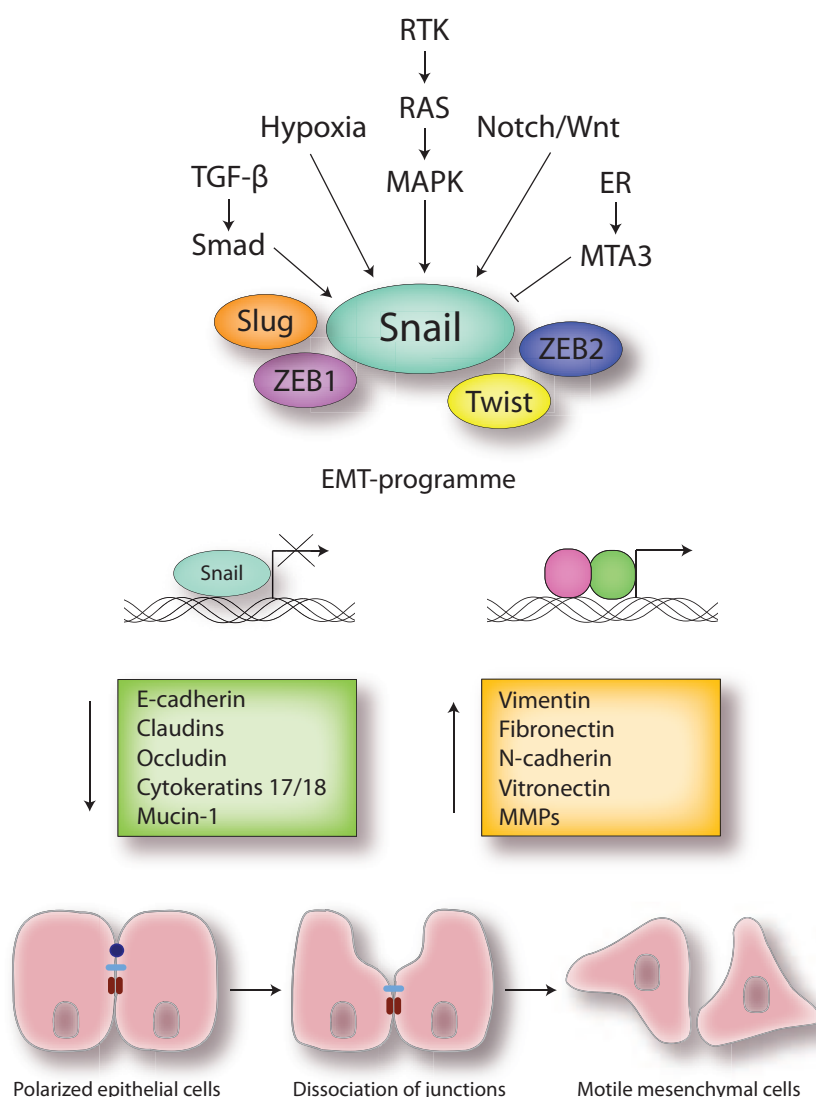


Figure 6. Schematic representation of the mechanisms regulating EMT. A number of signaling pathways resulting in repression of the E-cadherin gene transcription are involved in EMT-induction. Downregulation of E-cadherin and other epithelial-type specific proteins is mediated by different transcriptional repressors, such as Snail, and results in a transition from an epithelial to a mesenchymal phenotype. The EMT-programme includes upregulation of proteins important for invasion and metastasis, e.g. vimentin and MMPs.

The E-cadherin Repressor Snail

Snail is a highly unstable protein, which is degraded due to phosphorylation by glycogen synthase kinase-3 β (GSK-3 β). In addition, GSK-3 β regulates subcellular localization of Snail, and by phosphorylation of a consensus motif, distinct from the one involved in ubiquitination, Snail is retained in the cytoplasm²⁴⁸. Furthermore, regulation of ER α is mediated by the direct binding of Snail to EREs of the promoter of ER target genes, resulting in transcriptional inactivation²⁴⁹. Several additional mechanisms and functional mediators involved in Snail transcriptional activity and localization have been described²³⁷. In breast cancer elevated Snail expression has been associated with a poor clinical outcome²⁵⁰⁻²⁵².

A number of reports describe an *in vitro* induction of Snail and Slug accompanied by decreased expression of E-cadherin, as well as enhanced invasive and migratory capacity in hypoxia²⁵³⁻²⁵⁶. These data imply a crucial role for hypoxia in the induction of EMT, favoring the progression and invasive properties of cancer cells. Moreover, hypoxia has been shown to regulate EMT via Notch-induced Snail expression, by direct binding of the intracellular domain of Notch to the Snail promoter. Notch also mediates recruitment of HIF-1 α to the lysyl oxidase (LOX) promoter, resulting in increased LOX transcription, which in turn stabilizes the Snail protein²⁵⁷.

Novel Theories on EMT and Cancer

Recent studies have demonstrated a direct link between EMT and stem cells, implying that EMT generates cells with stem cell properties. Ectopic expression of Snail or Twist can promote EMT in both non-transformed and transformed mammary cells, where it induces a stem cell marker expression profile as well as rendering cells able to self-renew. Furthermore, stem cell-like cells isolated from normal mammary glands or breast carcinomas show increased expression of EMT markers, such as Snail, Slug and Twist²⁵⁸. In accordance with this finding, cancer stem cells potentially generated by EMT have been identified at invasive regions of tumors²⁵⁹.

Notably, the relevance of EMT in cancer progression has been debated in recent years²⁶⁰⁻²⁶². EMT is considered to be a transient and reversible process and does not necessarily account for all the steps required for tumor progression via invasion and metastatic spread. Several studies describe a partial or incomplete EMT phenotype of advanced carcinomas, displaying some mesenchymal features, but with a retention of well-differentiated epithelial cell characteristics²⁶¹.

The Tumor Microenvironment and Metastasis

Tumor Metastasis

The primary cause of death for the majority of cancer patients is the development of metastatic disease²⁶³. In breast cancer, 10-15 % of patients develop distant metastasis within three years of diagnosis, and approximately 30 % will eventually be presented with recurrent, advanced or metastatic disease²⁶⁴. A series of discrete biological events characterizes tumor metastasis and these include the movement of tumor cells from the primary tumor to a distant site of the body²⁶⁵. The first criteria of metastasis is the invasion of surrounding tissue, and this involves changes in adherence to neighboring cells and to the ECM, proteolytic degradation of tissue, and finally enhanced cell motility. Invasion is facilitated by altering the expression of ECM-interacting integrins, immunoglobulin superfamily receptors and surface proteoglycans, as well as the cell-cell interacting proteins cadherins (E-cadherin to N-cadherin). Degradation of surrounding tissue is mediated by secretion of MMPs, cathepsins and heparanases^{266, 267}. Cell motility is induced by growth factor activation of RTKs, leading to interaction of the RTKs with integrins which in turn stimulates focal adhesion kinase (FAK)-Src complex formation. This complex-formation promotes cell motility via rearrangements of the actin cytoskeleton, and formation of lamellipodia that are crucial for forward movement. Following invasion the tumor cells need to enter the bloodstream or the lymphatics, arrest and adhere to the vessel wall and extravasate into the tissue at the new site. The last step of metastasis is colonization, an event strictly dependent on interactions with the microenvironment at the distant location²⁶⁵. Gene expression profiling has revealed candidate genes or gene signatures associated with metastasis, serving as putative prognostic factors in human cancers^{268, 269}.

Components of the Tumor Microenvironment

The microenvironment is an important feature influencing the development of cancer, via extensive communication between tumor cells and the surrounding stroma. Paracrine signaling mediated by a vast number of cytokines and growth factors that promote proliferation, invasiveness and metastatic potential is the foundation of tumor progression²⁷⁰. The stromal compartment is constituted by several distinct cell types, such as immune and inflammatory cells, fibroblasts and vascular cells, and the relative abundance of each cell type differs depending on the local tumor site²⁷¹.

These cell types have been observed at the invasive front of tumors, contributing to the leading edge and hence enhancing infiltration^{272, 273}. In order to create a permissive environment, cancer cells have the ability to alter their surroundings, and the stromal compartment of a tumor is referred to as reactive tumor stroma²⁷⁴. This reactive stroma promotes activation of a number of different cell types, including the cancer-associated fibroblasts (CAFs). CAFs are mesenchymal-like cells that share characteristics with smooth-muscle cells and fibroblasts, and these cells enhance tumor progression by creating a supportive microenvironment for the malignant cells. CAFs regulate ECM deposition, recruit inflammatory mediators and also play a role in the induction of EMT, which in turn promotes tumor invasion^{275, 276}. Interestingly, experimental models have shown that impaired fibroblast motility results in reduced growth and metastatic spread of tumor cells²⁷⁷. In addition to CAFs, another cell type recruited to the tumor stroma is the tumor-associated macrophage (TAM). TAMs are essential for stimulation of tumor cell invasion, migration and angiogenesis, and hence also contribute to the reactive stroma²⁷⁸.

The Pre-Metastatic Niche

A prerequisite for metastasis is successful dissemination of tumor cells or “seed”, and the influence of the “congenial soil” or tumor microenvironment at the metastatic site plays a crucial role in this complex series of events²⁷⁹. The bone marrow-derived hematopoietic progenitor cell (HPC) has in recent years been recognized as an essential initiator of tumor metastasis, priming distant tissues for tumor cell implantation. These cells are believed to make up a so called pre-metastatic niche, by preconditioning the microenvironment for promotion of tumor metastasis, even before tumor cells can be found^{280, 281}. Changes in the local tumor stroma induced by secreted signaling mediators direct recruitment of bone marrow-derived cells needed for development of new vessels. Interestingly, clusters of HPCs in normal tissue that are common sites of metastasis have been observed in breast cancer patients, even before histological evidence of tumor²⁸⁰. These findings imply a crucial role for metastatic priming and accordingly, a way of identifying patients likely to suffer disease recurrence. However, only 50 % of breast cancer patients presented with micrometastatic disease of the bone marrow eventually develop metastasis²⁸². This might be explained by tumor cell dormancy, which means that the malignant cells are quiescent, and need a stimulatory signal to re-enter the cell cycle.

In order to establish a bone metastasis, unlike spreading to any other organ, tumor cells need to induce bone resorption by engaging the specialized osteocytes, which promote degradation of this calcified tissue²⁸³. The milieu of signaling factors that promote cancer-initiation resembles the natural environment of hematopoietic stem cells (HSCs) and HPCs, and hence metastasizing to bone observed for various cancers is not surprising. Specific chemokine repertoires can predict tissue-specific tropism of tumor metastasis. Breast, ovarian, prostate and brain cancers are osteotropic

cancers, in which cancer cells home to the distant site in a CXCR4⁺-dependent manner²⁸⁴⁻²⁸⁷. The bone marrow has been described as an “educator” of tumor cells, rendering them more prone to survive and potentially also more aggressive²⁸⁸.

In addition to the bone marrow-derived HPCs, recruitment of bone marrow-derived mesenchymal stem cells (MSCs) to tumor stroma has lately been given a great deal of attention. The MSC is a pluripotent stem/progenitor cell, distinct from the hematopoietic lineage, which is responsible for the maintenance and regeneration of various connective tissues, residing predominantly in the bone marrow²⁸⁹. These cells have been suggested as a quiescent source of stem cells in a number of different tissues, and have been reported to promote metastasis²⁷⁸. Bone marrow-derived human MSCs enhanced tumor metastasis of weakly metastatic breast cancer cells in an experimental setting²⁹⁰.

Tumor Stroma and Clinical Aggressiveness

Genomic alterations in cancer-associated stromal cells have in recent years been demonstrated to predict clinical outcome in a number of different cancers. In one study, gene analysis of stroma isolated from DCIS and invasive breast cancers revealed frequent loss of 11q21-23, 3p14.2, 16q23-24 and 17q24, suggesting that genetic alterations of the stroma contribute to tumorigenesis²⁹¹. Further studies have confirmed the finding of stromal specific LOH in different tumor types²⁹²⁻²⁹⁴, as well as identifying stromal gene signatures, associated with tumor aggressiveness²⁹⁵⁻²⁹⁷. Genes commonly reported to be upregulated in stroma include ECM components and MMPs, genes that mediate stromal remodeling, and genes associated with hypoxia and TAM immune response. Several explanations to how genetic changes in the stroma can reflect tumor aggressiveness have emerged, and it has been suggested that a genetic co-evolution of tumor and adjacent stromal cells occurs. Two different hypotheses describing the co-evolution of tumor and stromal cells have been proposed; one suggests that transformation of stromal cells occurs first and result in proliferation of epithelial cells, whereas the other implies initial changes of epithelial cells followed by changes in the stroma²⁹⁸.

By considering the essential role of the tumor microenvironment in tumorigenesis, we might gain better insight into the complex pathological mechanisms that underlie cancer development. This could potentially improve treatment management for advanced disease and subsequently reduce mortality. The reactive tumor stroma is an attractive clinical drug target, and by disrupting this intricate signaling system and “normalizing” the stroma, tumor progression might be slowed down. This promising approach has been reported to reverse the progression of breast cancer cells *in vitro*²⁹⁹. Interestingly, both tamoxifen and letrozole has been shown to not only effect epithelial tumor cells but also act on stromal cells^{300, 301}.

Perspectives of Breast Cancer

Breast cancer is a highly heterogeneous disease, representing several distinct histological as well as genetic subtypes, and at the molecular level a vast number of genetic alterations distinguishing between patients have been identified. However, some characteristics of breast cancer known to be associated with poor prognosis or resistance to certain therapies can be used to guide the therapeutical management, and these include features such as histological grade, tumor stage and expression of ER and PR. In the past decade it has been recognized that the environment surrounding the tumor has to be taken into account in tumor biology, nevertheless, adding further complexity to the story. Without the influence of the microenvironment a cancer would not progress, and hence this aspect of tumorigenesis is crucial both in prognostics and for therapeutic implications. Another aspect is tumor hypoxia, and the knowledge on the complex mechanisms underlying hypoxia-induced tumor progression is constantly expanding. Moreover, in recent years significant advances in our understanding of the molecular mechanisms behind EMT and its importance in cancer have been made. Gaining mechanistic insights into basic cellular processes involved in tumorigenesis will bring us closer to improved treatment strategies, including individualized therapies and minimizing the side effects associated with cancer therapeutics. Recurrence of endocrine resistant disease is a major clinical challenge and therefore identification of drug targets, as well as selecting patients likely to benefit from certain treatments are important clinical anticipations.

The Present Investigation

Aims

The general objective of this thesis was to identify biomarkers with potential prognostic significance and predictive value regarding tamoxifen treatment response in primary breast cancer. An additional main aim was to investigate the regulation of epithelial-mesenchymal transition in hypoxic breast cancer cells.

Specific Aims

- Identification of biomarkers associated with *CCND1* gene amplification, with potential involvement in tamoxifen resistance in premenopausal breast cancer patients.
- Elucidating the impact of hypoxia on regulation of epithelial-mesenchymal transition in breast cancer cell lines, and determining the importance of Snail as a prognostic and treatment predictive marker in breast cancer.
- Delineating the putative role of β -arrestin1 in clinical aggressiveness and tamoxifen response in pre- and postmenopausal breast cancer patients.

Results and Discussion

Clinical Relevance of the Present Investigation: Identifying Putative Markers for Prognosis and Prediction of Tamoxifen Response in Breast Cancer Patients (Paper I-III)

Despite the extensive research in the field of breast cancer it persists as a critical health burden. The ongoing search for putative biomarkers aims to identify clinically useful tools, in anticipation of improving therapeutic management and increasing patient survival with both early-stage and advanced disease. Tamoxifen remains the first-line treatment option for premenopausal breast cancer patients, and the process of selecting patients susceptible to this form of adjuvant therapy constantly needs to be refined. Endocrine resistance is a major clinical challenge and by distinguishing patients, that based on expression of certain biomarkers, are unlikely to respond to anti-estrogens, the goal of individualized breast cancer therapeutics may be attained more readily. A number of criteria need to be fulfilled and extensive research is required to successfully discover a novel prognostic or treatment predictive marker.

Each study of the present investigation has a diverging main focus, but they all attempt to elucidate the importance of identifying putative biomarkers, to gain a more comprehensive understanding of breast tumor biology and potentially identify patients unlikely to benefit from treatment with adjuvant tamoxifen. The analyses performed in paper I-III are retrospective studies of a patient cohort of 564 premenopausal breast cancer patients randomized to either two years of adjuvant tamoxifen or no adjuvant therapy. All patients included in the original prospective trial were presented with stage II invasive disease and were irrespective of hormone status randomly assigned to receive either tamoxifen or no adjuvant treatment. The original study aimed to compare survival between patients receiving tamoxifen and untreated patients. At present, the majority of breast cancer patients with invasive disease receive some kind of adjuvant therapy, making this patient cohort unique and a powerful tool in the search for treatment predictive markers. In paper III an additional breast cancer patient cohort was analyzed for expression of a specific biomarker. This cohort included 179 pre- and postmenopausal patients presented with invasive disease, and was designed as a screening cohort for antibodies in the Human Protein Atlas (HPA) program (detailed in paper III). Tumor tissue samples were available for 500 of the patient included in the randomized cohort and for all patients in the screening cohort. By employing the tissue microarray (TMA) technology, TMAs representing these patients had previously been constructed (Figure 7). The TMA technology is a commonly used method for high-throughput analyses of protein expression in large-scale tumor materials. For both tumor materials used in these studies two tissue core biopsies from each patient were retrieved from the donor paraffin block and transferred to the recipient block. Each recipient paraffin block contains tissue core biopsies corresponding to 50-100 patients. Extensive sectioning

of the paraffin blocks over time has resulted in a diminishing number of tissue cores in the large cohort, and hence a reduced number of patients were available for analysis. A slight over-representation of lobular cancers and cancers of low grade was observed among the missing cases, but regarding recurrence there was no difference between available and missing cases. Importantly, expression of the specific markers analyzed in these studies did not differ between the duplicate cores representing each patient, in the majority of cases. A major technical problem concerning studies describing potential biomarkers is the methodological discrepancy, including tissue processing, choice of antibodies and the statistical approaches used. In order to circumvent this issue certain guidelines for handling of biomarker studies was published in 2005³⁰². This study provides recommendations for study design and accurate presentation of data, to facilitate comparison of similar clinical studies.

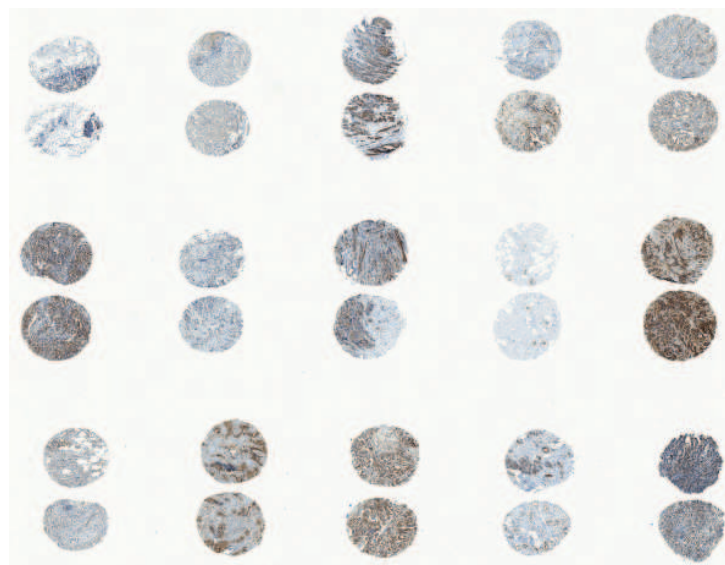


Figure 7. Representation of a TMA with tissue cores displaying different expression levels of the protein immunohistochemically stained for.

Loss of Chk1 is Associated with Amplification of CCND1 and Impaired Tamoxifen Response in Premenopausal Breast Cancer Patients (Paper I)

Amplification of chromosome 11q13 is a well-established cause of oncogenic activation, reported to occur in various cancers. The search for genes included in this genetic alteration that might be linked to clinical outcome has resulted in identification of a number of different markers, found at this chromosomal locus^{142, 156, 162}. The studies performed in paper I are based on the previous finding that *CCND1* amplification was associated with an agonistic effect of tamoxifen in the randomized patient cohort. Elevated protein expression of cyclin D1 conferred tamoxifen resistance in these patients despite ER-positivity, but the agonistic effect was exclusively observed in patients exhibiting the amplification. Speculatively, altered protein expression of an additional marker associated with the amplification might be responsible for the

agonistic action of tamoxifen. In order to characterize putative markers suitable for immunohistochemistry (IHC) analysis, we selected three distinct genes known to be involved in the 11q13 amplification event; *CTTN*, *FADD* and *CHK1* (loss of distal 11q), based on an array comparative genomic hybridization (CGH) screen. Array CGH is a molecular cytogenetic technique that offers a systematic approach to obtaining profiles of chromosomal aberrations within single tumor specimens. The microarrays employed contain large insert size genomic clones and hence a genome-wide search for genes exhibiting altered copy number levels can be performed.

For this study protein analyses were performed in the randomized cohort. Following antibody validation, IHC was employed for each marker and stainings were assessed based on staining intensity (cortactin and FADD: cytoplasmic and Chk1: nuclear), whereas for Chk1 an additional evaluation of percentage positive nuclei was made. Protein expression was scored as 0-3, based on staining intensity, for all three markers and as 0-5, 6-10, 11-25, 26-50 and 51-100 % positive nuclei for Chk1. For subsequent statistical analyses staining intensity was subcategorized as low (0-1) and high (2-3) for cortactin and FADD. Data for cyclin D1 and Pak1, which were also included in the analyses, had been evaluated in previous studies^{93, 104}. Cyclin D1 nuclear intensity was also assessed as low versus high, whereas for Pak1 nuclear positivity was evaluated, scored as negative or positive nuclei. For Chk1 which predominantly resides in the nucleus the variable of positive nuclei, further subcategorized as 0-5, 6-50 and 51-100 %, was used for the statistical analyses.

The outcome of the statistical analyses performed revealed an association for all markers, including Pak1, with *CCND1* amplification, supporting the hypothesis that co-amplification of *CTTN*, *FADD* and *PAK1* with *CCND1* could also be represented at the protein level. Furthermore, Chk1 protein expression inversely correlated to *CCND1* amplification, confirming the frequent loss of distal 11q concurrently with 11q13 amplification. High expression of Chk1 was associated with a more aggressive breast cancer phenotype, defined by tumor size, grade and proliferation rate, in line with previous findings¹⁷⁷. However, Chk1 expression had no impact on recurrence-free survival (RFS) in the patient cohort. High FADD expression, but not high expression of cortactin, was linked to a reduced RFS in untreated patients. Moreover, expression of neither cortactin nor FADD was associated with an altered tamoxifen response. In order to specify loss of Chk1 expression a new subcategorizing in which patients representing low Chk1 expression as well as accurate expression of the proliferation marker Ki67, constituted one subgroup. The intimate link between cell cycle activity and checkpoint induction was confirmed by a positive correlation between Chk1 and Ki67, implying low checkpoint activity as proliferation is low. Accordingly, the new Chk1 parameter was constructed to exclude false negative Chk1 low patients, exhibiting low Chk1 expression as a result of low proliferation. The new variable was employed in the survival analyses, with the anticipation to better represent *CHK1*

loss. Chk1 expression, defined as normal or deviant (low Chk1 expression and intermediate Ki67 expression), based on the new subgroups, was found to impact upon tamoxifen response. In patients with tumors of deviant Chk1 expression the effect of tamoxifen was impaired, implying that loss of the *CHK1* gene may identify patients less likely to respond to this form of adjuvant therapy. However, the agonistic effect of tamoxifen observed for patients exhibiting *CCND1* amplified tumors was not due to loss of Chk1 expression.

The biological mechanisms rendering patients with tumors of low Chk1 expression insensitive to tamoxifen are unknown and no previous studies have to our knowledge identified Chk1 to be involved in tamoxifen responsiveness. However, loss of 11q24 has been associated with reduced tamoxifen sensitivity in breast cancer cell lines, implying presence of putative treatment predictive markers in this chromosomal region³⁰³. Moreover, the previously mentioned gene signatures described as predictors of tamoxifen sensitivity often include genes associated with cell cycle control. The importance of Chk1 in the cell cycle checkpoints suggests a potential role of this protein in endocrine resistance caused by deregulation of the cell cycle machinery. A recent report suggests a link between ER signaling and the DNA damage response pathway, demonstrating an inhibitory effect of estrogen on ATR activity, via plasma membrane-localized ER α in a breast cancer cell line and mouse mammary epithelial cells³⁰⁴. In addition, the protein association between Chk1 and claspin was blocked by estrogen, mediated by AKT phosphorylation of Chk1, preventing Chk1 signaling to the G2/M checkpoint. Interestingly, treatment with fulvestrant significantly counteracted the action of estrogen. These results imply a novel role for estrogen in chromosomal instability, through bypassing of the G2/M checkpoint induced by DNA damage, potentially relevant in breast cancer. Speculatively, functional DNA damage response machinery is required for tamoxifen to actively suppress proliferation. In tumors displaying an aberrant checkpoint pathway ER-inhibition by tamoxifen may have an insignificant effect in contrast to in tumors with functional repair machinery. It is however likely that several pathways are involved in tamoxifen resistance caused by defective Chk1 function. Moreover, the balance between signaling via nuclear and plasma membrane-localized ERs potentially affects the response to this adjuvant therapy. Since Chk1 expression was only shown to be associated with tamoxifen resistance in univariate analysis, our results should be interpreted with caution. Further comprehensive studies desirably including randomized breast cancer cohorts are required to support these results. Nevertheless, the involvement of distal 11q loss in breast cancer progression and treatment sensitivity is potentially as crucial as amplification of 11q13, and should be considered as a putative target for further characterization.

Hypoxia Induces a Partial EMT in vitro and the Key Regulator Snail Predicts Tamoxifen Sensitivity in Primary Breast Cancer (Paper II)

EMT has in recent years been recognized to play a crucial role in metastasizing of solid tumors, and a wealth of reports investigating the biological mechanisms behind this phenomenon has been published. Tumor hypoxia is a cellular process demonstrated to induce EMT, and hypoxic regulation of key E-cadherin repressors such as Snail and Slug has been the main focus of many studies, elucidating the pathological activation of EMT observed in cancer^{254, 255}. This study in line with paper I and III aimed to elucidate the prognostic and treatment predictive significance of a specific biomarker, crucial for induction of EMT, as well as studying regulation of EMT in the context of hypoxia in a panel of human breast cancer cell lines. A limiting factor in this field of research is the discrepancy between studies reporting prognostic significance for EMT-regulators such as Snail. The use of unspecific antibodies and inconsistent evaluation of IHC are bias affecting the accuracy of this kind of studies.

For the analyses in paper II a substantial validation of the Snail antibody was undertaken, to achieve accurate and representative results both regarding IHC analyses and the *in vitro* studies. The induction of EMT by hypoxia was investigated, with main focus on the key E-cadherin transcriptional repressor Snail. Initially, the four breast cancer cell lines MCF-7, T-47D, MDA-MB-468 and MDA-MB-231 were exposed to hypoxia for increasing time points and the expression of E-cadherin, vimentin and Snail was monitored by Western blot and immunocytochemistry (ICC), as well as the migratory propensity. We found that Snail expression was elevated as breast cancer cell lines were exposed to hypoxia, with changes also observed for protein levels of the epithelial marker E-cadherin and the mesenchymal marker vimentin, in some of the cell lines. Surprisingly, hypoxia only enhanced the migratory propensity in one of four cell lines (MDA-MB-468), in which vimentin expression increased and E-cadherin expression was unaffected by hypoxia. This led us to the conclusion that EMT was only partially induced as a result of hypoxic exposure *in vitro*. Furthermore, hypoxia was shown to regulate the subcellular localization of the Snail protein, increasing the nuclear protein expression as a response to prolonged exposure. The nuclear localization was related to the time point when expression changes of E-cadherin and vimentin started to become evident in the cell lines. Next, we wanted to examine the role of Snail in cell migration, by overexpressing or silencing the protein prior to migration analysis, in the cell lines. Snail overexpression promoted migration of MCF-7, T-47D and MDA-MB-231 cells, whereas silencing resulted in reduced migratory capacity of MCF-7, MDA-MB-468 and MDA-MB-231 cells. Surprisingly, the migratory phenotype induced by Snail appeared to be independent of E-cadherin and vimentin expression.

In order to analyze the regulation of EMT induced by hypoxia in an *in vivo* model system, full sections of DCIS were stained with Snail antibody, and compared with stainings of HIF-1 α , E-cadherin and vimentin on corresponding sections. Snail

expression was found to be elevated in hypoxic areas of DCIS, but no changes were observed for E-cadherin and vimentin levels in these regions, further suggesting an incomplete induction of EMT in non-invasive breast cancer. Our next objective was to elucidate the putative role of Snail in breast cancer progression and tamoxifen sensitivity. The TMAs from the randomized cohort of breast cancer patients were stained with Snail antibody by IHC. Staining was evaluated and scored as negative or positive nuclei, based on observation that cytoplasmic staining was detected at a relatively constant level between samples. Interestingly, when analyzing Snail expression in relation to clinico-pathological parameters nuclear Snail expression was associated with an aggressive tumor phenotype defined as high grade and high proliferation rate, as well as with the hypoxia marker CAIX, supporting the association between EMT and hypoxia. Furthermore, nuclear Snail was inversely correlated to ER and PR expression, confirming previous findings. However, no significant independent prognostic information for Snail was retrieved from the analyses.

The observations that Snail directly represses ER gene transcription in breast cancer cells and that Snail has been reported to be negatively correlated to expression of ER in clinical materials ²⁴⁹, motivated us to elucidate the potential involvement of Snail in the tamoxifen response. Accordingly, we examined whether the effect of tamoxifen treatment was modified by Snail by including an interaction term to the multivariate Cox regression model. In a multivariate analysis the relationship between multiple parameters is analyzed to identify those with a dominant effect on outcome (independent predictors of outcome). The RFS was compared between treated and untreated patients according to Snail expression. Interestingly, Snail was found to significantly compromise the effect of tamoxifen, rendering patients with tumors exhibiting no nuclear Snail expression resistant to this adjuvant therapy. Consequently, Snail is a putative marker predicting response to tamoxifen, a novel finding that might be of clinical relevance for future therapeutical implications. Underlying mechanisms to reduced tamoxifen sensitivity in Snail-negative cancers are unexplored, but the relationship between Snail and the ER might play a role. Hypothetically, in a cancer cell completely lacking nuclear Snail, ER transcriptional activity would be higher, suggesting that the effect of tamoxifen might not sufficiently repress the action of estrogen. Additionally, Snail has been shown to suppress estrogen production, via inhibition of aromatase, resulting in reduced levels of circulating estrogen ³⁰⁵. Hence, lack of Snail expression would result in an excess of estrogen, potentially binding to ER and competing with tamoxifen. However, a plethora of factors and signaling pathways are involved in endocrine resistance and the context in which all these mediators act determines whether a cancer cell is sensitive or insensitive to drugs like tamoxifen. It is therefore relevant to screen for potential biomarkers involved in therapeutic resistance, and when these have been identified, continue to study the putative biological processes these markers might be involved in.

Expression of Stromal β -arrestin1 Predicts Clinical Outcome in Breast Cancer Patients (Paper III)

The role of the GPCR adaptor protein β -arrestin1 in cancer has to date not been extensively studied. However, β -arrestin1 has been implicated in cell motility *in vitro*, as well as in metastasis formation in an experimental model^{191, 192}. Its corresponding gene *ARRB1* maps to chromosome 11q13 and hence might be involved in the genetic events frequently occurring at 11q. In paper III the potential involvement of β -arrestin1 in amplification/deletion of chromosomal regions at 11q, and the role as a putative biomarker associated with disease outcome and treatment response was investigated. Based on the findings that cyclin D1 and Pak1 (11q13), as well as Chk1 (11q24) in paper I, were linked to tamoxifen resistance we attempted to elucidate a potential association between β -arrestin1 and treatment response. Moreover, the importance of β -arrestin1 in a clinical setting had not previously been reported.

In order to examine the expression of β -arrestin1 in breast cancer two breast cancer patient cohorts were analyzed for β -arrestin1 staining; the randomized cohort previously described and the screening cohort including both pre- and postmenopausal patients. The initial analysis revealed an unexpected expression pattern of β -arrestin1 showing staining of both tumor cell cytoplasm and cells of the stromal compartment of the cancerous tissue. Accordingly, both tumor cell and stromal staining was evaluated, and staining intensity was scored as 0-3 for both compartments. Staining intensity of tumor cells versus stromal cells varied significantly between tumor samples, and tumors of high tumor cell intensity and low stromal intensity, as well as the opposite, was frequently observed. However, in the majority of tumors staining intensity of the two compartments was similar, with no variation or a variation of one score between tumor and stromal cells. Our first objective was to analyze the expression of β -arrestin1 in relation to general clinico-pathological variables. Interestingly, tumor cell and stromal β -arrestin1 expression was associated with separate sets of tumor characteristics in both cohorts. High β -arrestin1 expression in tumor cells was associated with ER and PR (cohort II) negativity and amplification of *HER2*, whereas high stromal expression was associated with higher grade (cohort I and II), larger tumors and higher proliferation rate (cohort II), as well as lymph node involvement and distant metastasis (cohort I). This discrepancy implies distinct functionalities of β -arrestin1 in tumor cells and the surrounding stromal cells in the context of clinical aggressiveness in breast cancer. Next, the impact of β -arrestin1 on RFS was analyzed in both cohorts. In line with the previous results showing a stronger association of stromal protein expression to clinico-pathological parameters defining aggressive disease, RFS was affected by stromal β -arrestin1 but not by tumor cell expression. In patients presented with tumors of high stromal β -arrestin1 expression, time to recurrence was shorter compared to in patients with tumors of negative to moderate expression in cohort I. Surprisingly, in cohort II both high and negative stromal expression was associated with reduced RFS, suggesting that intermediate levels of β -arrestin1 is preferential to lack of or excessive

expression. In order to further examine the prognostic impact of β -arrestin1 we performed multivariate analyses for both cohorts. In cohort I β -arrestin1 revealed a prognostic significance, independent of other clinico-pathological parameters, thus representing a prognostic marker for breast cancer recurrence. Subcategorizing of patients displaying negative or high expression of β -arrestin1 versus low or moderately-expressing in cohort II, was also significantly predictive of prognosis. Patients with tumors of negative or high expression were subjected to a shorter RFS compared to patients exhibiting low or moderate expression. Furthermore, expression of β -arrestin1 did not modify the effect of tamoxifen. The novel discovery of stromal β -arrestin1 expression as a predictor for clinical outcome in breast cancer was however intriguing. Alterations in the stromal compartment of a tumor may as previously reported have an impact on malignant progression.

A potential mechanism explaining the association between high stromal β -arrestin1 expression and worse prognosis, may be the recently described role of β -arrestin1 in growth factor receptor signaling. Moreover, β -arrestin1 has been reported to have a dual role in IGF-1R signaling, both regulating IGF-1R degradation and mediating IGF-1 induced MAPK signaling, potentially supporting the observation that patients with tumors displaying either no expression or high levels of β -arrestin1 are subjected to a worse clinical outcome^{306, 307}. However, further studies investigating the involvement of β -arrestin1 in growth factor receptor signaling and tumor progression are required to determine the putative prognostic significance of this protein in breast cancer.

Conclusions

In this thesis we have identified prognostic and treatment predictive markers in breast cancer, as well as gained insights into the hypoxic regulation of EMT, and the importance for the key regulator Snail in cell motility.

We could conclude that:

- Loss of Chk1 protein expression is associated with amplification of the *CCND1* gene and confers tamoxifen resistance in premenopausal breast cancer patients.
- Hypoxia induces an incomplete EMT *in vitro*, and expression of Snail impacts upon the migratory capacity of breast cancer cell lines.
- Absence of nuclear Snail expression renders breast cancer patients less sensitive to adjuvant tamoxifen.
- Stromal β -arrestin1 is a putative prognostic marker in pre- and postmenopausal patients, but is not linked to an altered tamoxifen sensitivity.

Populärvetenskaplig Sammanfattning

Många av oss har en närstående, släkting eller bekant som fått diagnosen cancer. Risken att som svensk drabbas av cancer innan 75 års ålder är nästan 30 procent. Hos kvinnor är den vanligaste formen bröstcancer, vilken drabbar ungefär en av tio svenska kvinnor. Trots att allt fler insjuknar i bröstcancer har dödligheten sjunkit de senaste decennierna, mycket tack vare införandet av mammografin och nya, mer effektiva behandlingsmetoder.

Det är viktigt att komma ihåg att bröstcancer är en komplex sjukdom med väldigt individuell sjukdomsbild från patient till patient. Bröstcancer behandlas med kirurgi, då antingen hela bröstet eller bara tumören med en del omgivande vävnad avlägsnas, beroende på tumörens omfattning. Ofta strålas patienten som ett komplement till kirurgin, för att döda eventuella resterande cancerceller. Flertalet patienter får även en tilläggsbehandling, så kallad adjuvant behandling, vilken utgörs av kemoterapi eller anti-hormonell terapi. Ungefär 70 procent av alla patienter med bröstcancer har tumörer som uppvisar östrogenreceptorn och dessa patienter utgör den grupp som behandlas med anti-östrogenterapi. Eftersom östrogen stimulerar tumörtillväxt kan man få tillväxten att avstanna genom att blockera antingen östrogenreceptorn eller produktionen av östrogen. Den allra vanligaste formen av anti-östrogenbehandling är preparatet tamoxifen, vilket interagerar med östrogenreceptorn och upphäver östrogenets effekt.

Trots att majoriteten av alla bröstcancerpatienter har tumörer där östrogenreceptorn finns i cancercellerna, har tamoxifen bara effekt hos ungefär hälften av patienterna. För de resterande patienterna är denna behandlingsform helt verkningslös. Detta fenomen benämns som tamoxifenresistens och är ett omfattande problem inom bröstcancerbehandlingen. Det är därför viktigt att kunna identifiera patienter som inte kommer att bli hjälpta av behandling med tamoxifen, så att de inte behandlas i onödan. Ett antal biologiska mekanismer har beskrivits som potentiella förklaringar till resistens, men fortsatt forskning som inriktar sig på de bakomliggande orsakerna till uppkomsten av tamoxifenresistens är nödvändiga. Ytterligare ett viktigt mål med forskningen är att finna markörer, dvs. specifika proteiner som kan förutsäga ett försämrat behandlingsvar. Idag finns få kliniska markörer som används för att avgöra om patienten bör behandlas med tamoxifen eller inte. Markörer som används för att förutse hur en viss typ av behandling kommer att påverka sjukdomsutfallet

kallas för prediktiva markörer, och för att förutspå anti-hormonellt behandlingsvar används förekomsten av östrogenreceptorn som en prediktiv markör. För att kunna hindra utvecklingen av en tumör, och för en bättre vägledning i val av rätt behandlingsmetod, är det av stor betydelse att finna prediktiva markörer såväl som markörer som kan förutse sjukdomsförloppet, vilka benämns som prognostiska markörer. Målsättningen med den här avhandlingen var att identifiera prediktiva såväl som prognostiska markörer i bröstcancer, utöver de som används inom sjukvården idag, för att eventuellt kunna bidra med viktig information gällande tamoxifenresistens och sjukdomsbild i framtiden.

Genom att studera bröstcancerpatienter som behandlats med tamoxifen och jämföra deras överlevnad med den för obehandlade patienter, har vi kunnat ta reda på vilka patienter som svarar på behandlingen och vilka som inte gör det. Det patientmaterial som vi gjort våra analyser i baserar sig på en tidigare utförd klinisk studie, och är unikt i det avseendet att hälften av patienterna behandlats med tamoxifen och den resterande hälften inte med någon adjuvant behandling alls. Idag behandlas de flesta kvinnor med bröstcancer med någon form av adjuvant behandling, varför den här typen av studie inte skulle kunna upprepas. Vi har studerat förekomsten av specifika proteiner hos dessa patienter och har kunnat dra slutsatsen att uttryck av dessa proteiner dels kommer att påverka hur det går för patienterna, dels om de är mottagliga för tamoxifenbehandling eller inte. Vi hade möjlighet att analysera tumörvävnad från dessa bröstcancerpatienter, med hjälp av en teknik där man konstruerar så kallade vävnadsarrayer. En vävnadsarray består av små biopsier (vävnadsprover) som ordnas i ett system som gör det möjligt att analysera biopsier från ungefär 100 patienter på ett och samma objektglas eller vävnadschip. Analyserna bygger på att de specifika proteiner som vi ville studera färgas in på dessa vävnadschips, med en teknik som kallas immunhistokemi. Följaktligen kan uttrycket av proteinet bedömas i mikroskop och vi kunde göra statistiska jämförelser mellan patienterna. Det går även att se hur uttrycket av proteinet är kopplat till för varje patient redan kända karakteristika som t.ex. tumörstorlek, grad av tumöraggressivitet och om patienten hade en östrogenreceptorpositiv tumör.

Ett viktigt kännetecken för cancerceller är att de har drabbats av genetiska förändringar. Genetiska förändringar kan uppstå som följd av en rad olika mekanismer, som t.ex. mutationer, förlust av delar av en kromosom, eller ett ökat antal kopior av en och samma gen. Ett ökat antal kopior av en viss gen uppstår genom en process som kallas genamplifiering, och involverar ofta ett antal olika gener som är belägna i närheten av varandra på en kromosom. Kromosom 11q13 är en region som ofta amplifieras i olika former av cancer och i detta kromosomavsnitt finns genen för proteinet cyklin D1. I bröstcancer har amplifiering av genen cyklin D1 visat sig leda till att tamoxifen kan stimulera istället för att hämma tumörtillväxt. I 15 procent av alla cancerfall med cyklin D1-amplifiering är också proteinmängden av cyklin D1 förhöjd i cancercellerna. Ett ökat uttryck av cyklin D1-proteinet har visat sig vara kopplat till ett sämre svar på tamoxifenbehandling, men inte till en motsatt (tumörfrämjande) effekt, som vid

genamplifiering. Flera olika gener är involverade i amplifieringen av kromosom 11q13 och i delarbete I undersökte vi några olika gener belägna på kromosom 11, för att eventuellt hitta ytterligare en gen som kan ha ett samband med en omvänd effekt av tamoxifen. Genen för Chk1 förloras ofta i samband med att cyklin D1-genen amplifieras, och genom att studera proteinuttrycket av Chk1 kunde vi visa att om en bröstcancerpatient förlorat uttrycket av detta protein, så kommer tamoxifen att ha en betydligt sämre effekt än om proteinet uttrycks på en normal nivå. Den motsatta effekt av tamoxifen som observerats hos patienter med cyklin D1-amplifiering var dock inte beroende av Chk1.

Syrebrist, även kallat hypoxi, påträffas ofta i bröstcancertumörer och har ett samband med graden av aggressivitet. En hypoxisk tumör är associerad med en sämre prognos än en väl syresatt tumör. Hypoxi har visat sig framkalla något som kallas epitelial-mesenkymal transition (EMT), vilket kännetecknas av en mer aggressiv cancer, som också är mer benägen att metastasera (sprida sig). I delarbete II var målsättningen att undersöka om hypoxi leder till EMT i ett antal olika typer av odlade bröstcancer-celler. Vi kunde dra två slutsatser; dels att hypoxi bara delvis leder till EMT i bröstcancer-celler, dels att förekomsten av proteinet Snail, som reglerar EMT, påverkar hur rörlig en cancer-cell är. Dessutom identifierade vi Snail som en potentiell prediktiv markör för hur väl tamoxifen fungerar. Hos patienter som saknade Snailproteinet i tumörcellernas kärna hade tamoxifen betydligt sämre effekt än hos patienter vars tumörer uppvisade proteinuttryck av Snail. Avsaknad av Snail i tumörcellkärnan är alltså en indikation på tamoxifenresistens.

För att en tumör ska kunna växa är den beroende av sin omgivning, som kallas tumörstromat. En ömsesidig signalering sker ständigt mellan tumörcellerna och stromat, vilket leder till en fördelaktighet som innebär att tumörcellerna blir mer aggressiva. Längre ansågs det att genetiska förändringar enbart sker i cancer-celler, men det har nyligen blivit vedertaget att genetiska förändringar kan förekomma även i stromat. Ett genetiskt förändrat tumörstroma har dessutom visat sig vara kopplat till en sämre prognos, med elakare tumörer som är mer benägna att metastasera som följd. I delarbete III undersökte vi hur uttrycket av proteinet β -arrestin1, som också har sin motsvarande gen på kromosom 11q13, är relaterat till tumöraggressivitet. Förutom det tidigare nämnda patientmaterialet använde vi oss av ytterligare ett material som enbart inkluderade behandlade bröstcancerpatienter. Våra resultat visade att ett högt uttryck av β -arrestin1 i celler från stromat, men inte i själva tumörcellerna, var tecken på en dålig prognos för patienten. Intressant nog visade det sig att fullständig avsaknad av β -arrestin1 också var kopplat till en sämre prognos i det första patientmaterialet, vilket tyder på att en intermediär (mellanliggande) nivå av detta protein är att föredra jämfört med ett för lågt eller ett för högt uttryck i de stromala cellerna. Följaktligen kan β -arrestin1 möjligen användas som en prognostisk markör i bröstcancer, men till skillnad från Chk1 och Snail var β -arrestin1 ingen prediktiv markör för effekten av tamoxifen.

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