IDENTIFYING GENES RESPONSIBLE FOR TAMOXIFEN RESISTANCE IN BREAST CANCER

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Het identificeren van genen die verantwoordelijk zijn voor tamoxifen resistentie in borstkanker

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CHAPTER 1

GENERAL INTRODUCTION

Breast cancer is one of the leading causes of death of women in western countries. It affects one out of eight females in the USA (1) and one out of nine females in The Netherlands (www.kankerregistratie.nl) during their lifetime. Many risk factors for breast cancer have been identified including gender, familial susceptibility, age, and exposure to hormones i.e. use of exogenous hormones, young age at menarge, and high age at menopause and first pregnancy (2). Familial breast cancer accounts for 5-10% of total breast cancer. The remaining 90-95% are called "sporadic". Occasionally breast cancer also affects males (1% of the breast-cancer incidence in women).

In The Netherlands there are approximately 12000 new cases and about 3300 deaths yearly as a result of the disease. Since 1994, the mortality has slightly decreased due to earlier detection, following the introduction of the national breast cancer-screening program, and better treatment strategies (http://www.rivm.nl). Breast cancer patients may be subjected to various treatments including surgery, radiation, chemotherapy, molecular targeted therapy, or endocrine (hormonal) therapy. Often treatment strategies are combined. Surgery forms a part of nearly every patient's treatment for breast cancer, resulting in partial removal (lumpectomy) or total removal of the breast (mastectomy). Radiation may be used before or after surgery, and may accompany chemotherapy. In molecular targeted therapy, compounds like monoclonal antibodies or small tyrosine kinase inhibitors inhibit a specific target molecule. In contrast to conventional chemotherapy, which acts on all dividing cells generating toxic effects and damage of normal tissues, targeted drugs allow to hit, in a more specific manner, subpopulations of cells directly involved in tumor progression. Endocrine therapy works by interfering with the estrogen pathway that enhances cell-proliferation. It is applied for prevention, adjuvant therapy, and for treatment of metastatic cancers in patients with hormone receptor-positive tumors (3, 4).

ESTROGENS AND THEIR RECEPTORS

Estrogens are important regulators in the development of the normal breast, but also in breast cancer expansion and progression. More than a century ago, Scottish surgeon George Beatson noted that removal of the ovaries, which later was proven to cause reduction of endogenous estrogen levels, could induce breast cancers to regress. (5). In premenopausal women endogenous estrogens mainly originate from the ovaries. In postmenopausal women peripheral aromatization of adrenal androgens is their main resource. Estrogens perform their function by interacting with the estrogen receptors (ER). Two ER genes have been identified in mammals, ERα and ERβ, which show similar DNA- and ligand-binding properties, but distinct tissue distributions and functions (6-9). In ER signaling, there are several mechanisms of action: classical, ERE-independent,

ligand-independent, and nongenomic (Figure 1.1) (10-12). In the classical mechanism, upon ligand binding, the cytoplasmic receptor is translocated to the nucleus were it dimerizes, recruits co-activator proteins and general transcription factors, and activates genes with an estrogen responsive element (ERE). Most of these genes are involved in cell proliferation and survival or in maintaining tissue architecture (13-15). Ligand-bound ER can also modulate gene expression through interaction of the receptor with Fos and Jun at activator protein-1 (AP-1) binding sites (16), or with GC-box bound specificity protein 1 (SP-1) (ERE-independent) (17). In the ligand-independent mechanism, ER is phosphorylated by growth factors or signaling molecules leading to dimerization, DNA binding, and activation of transcription (18, 19). Activation of a membrane-associated form of ER causes rapid nongenomic effects of estrogen signaling (20, 21). In breast cancer ERα is highly expressed, which results in enhanced proliferation without differentiation or apoptosis. In contrast, expression of ERβ declines with increasing tumor

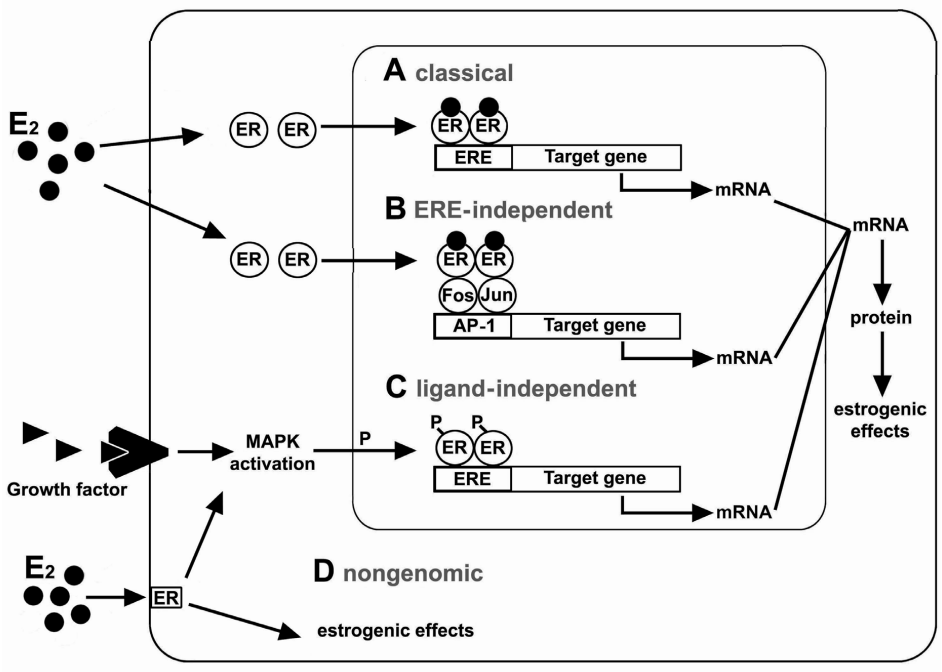

Figure 1.1 At least four mechanisms of estrogen-receptor (ER) signaling are known. A, the genomic, ligand-dependent, classical mechanism; Estradiol (E2)-bound ER dimers bind to EREs in target promoters, resulting in an up- or downregulation of gene transcription and subsequent estrogenic effects in the cell. B, the genomic, ligand-dependent, ERE-independent mechanism; Ligand-bound ER complexes bind to alternative response elements such as AP-1 through association with other DNA-bound transcription factors like Fos and Jun, upregulating gene transcription. C, the genomic, ligand-independent mechanism; Growth factors or signaling molecules (not shown) activate intracellular kinase pathways leading to phosphorylation (P) and activation of ER and subsequent target-gene transcription in a ligand-independent manner. D, the nongenomic, ligand-dependent mechanism; E2 binds to a possible membrane-associated form of ER, directly activating intracellular signaling cascades that generate rapid estrogenic effects in the cell. For simplification the roles of cofactors in ER signaling have been left aside in this figure. (Based on Figure 1 from ref 10)

aggressiveness (11). Nearly 70% of breast cancers are ERα positive and may be eligible for endocrine therapy.

THERAPEUTIC AGENTS FOR ENDOCRINE THERAPY

The estrogen dependence of breast cancer represents a unique feature of the disease that can be manipulated to prevent tumor development and effectively control growth. The current strategy for treatment of hormone-dependent breast cancer is to inhibit estrogen synthesis or block the action of estrogen by ovarian ablation or application of endocrine therapy using selective estrogen receptor modulators (SERMs), selective estrogen receptor downregulators (SERDs), luteinizing hormone-releasing hormone (LHRH) agonists or aromatase inhibitors (AIs) (Figure 1.2) (3, 22-24)

SERMs

SERMs are therapeutic agents functioning in a tissue-specific manner. They are agonists in some tissues, such as the endometrium and bone, and antagonists in others, like breast (25). The SERM tamoxifen has been the mainstay of endocrine therapy of breast

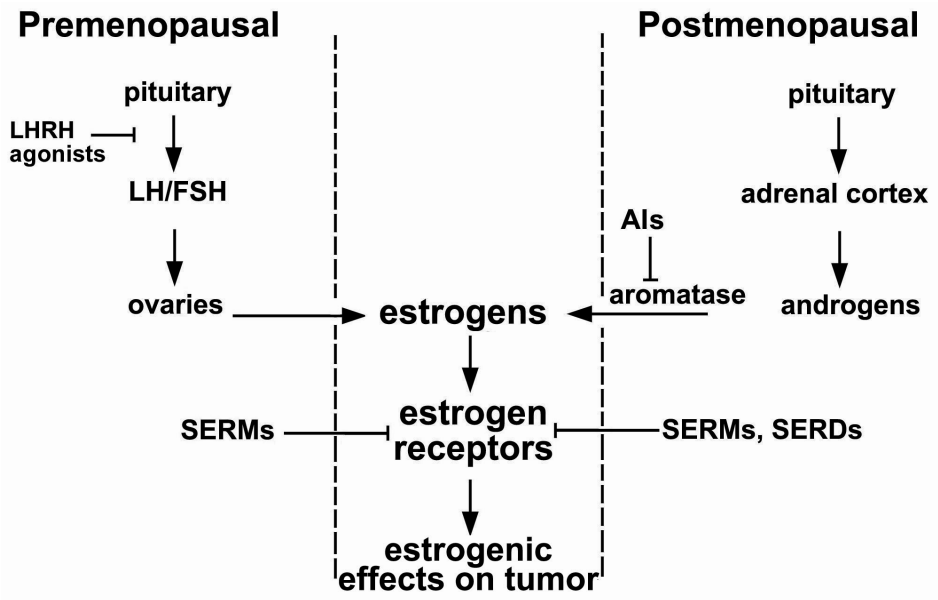

Figure 1.2 Various classes of endocrine agents are used to treat ER positive breast cancer. In premenopausal women LHRH agonists desensitize the hypothalamus-pituitary-ovarian axis blocking the production of estrogens by the ovaries. In postmenopausal women aromatase inhibitors (AIs) prevent the conversion of androgens into estrogens in peripheral tissues. SERMs and SERDs prevent ER-mediated cell replication by binding to or degrading the ER.

cancer for more than 25 years. It is able to bind the ligand-binding domain of the estrogen receptor, altering the molecular conformation of the receptor. Usually co-repressor instead of co-activator proteins are recruited, resulting in inhibition of transcription and subsequent tumor growth. Tamoxifen improves survival in patients with early breast cancer (26) and improves the quality of life when used as palliative treatment (27). Due to its agonistic activity, tamoxifen slightly preserves bone density. However, it is also associated with an increased risk of thromboembolic events (28) and endometrial cancer (29) in postmenopausal women. To reduce some of the harmful effects of tamoxifen other SERMs, like raloxifene, have been developed. Raloxifene has been shown to be equally effective as tamoxifen in reducing the risk of invasive breast cancer and has less side-effects. However, for treatment of premenopausal women the efficacy and safety of raloxifene have not been examined extensively enough yet (30).

Besides decreasing breast-cancer recurrence when used for adjuvant therapy, SERMs also play a role in breast-cancer prevention. Tamoxifen is the only drug approved for chemoprevention of breast cancer. In high-risk premenopausal and postmenopausal women it decreases breast-cancer incidence by 30-50% (29, 31-34). The main concern about using tamoxifen as a chemopreventive agent in postmenopausal women, are the side-effects (29, 34), an increased risk of thromboembolic events (28) and endometrial cancer (29). However, these side effects were less severe in premenopausal women (4, 35), who therefore have a favorable risk-benefit ratio for using tamoxifen in chemoprevention.

SERDs

Upon ER binding SERDs, such as fulvestrant (Faslodex/ICI 182,780) (Astrazeneca), inhibit receptor dimerization and abrogate estrogen signaling. Fulvestrant may be beneficial in the treatment of ERα-expressing tamoxifen-resistant tumors (22, 36). It has no demonstrable agonistic activity and therefore it does not increase blood-cloth and endometrial-cancer risk, nor preserves bone density (22). Fulvestrant is mainly applied as a therapy for postmenopausal women. Recent findings, however, suggest a possible benefit for premenopausal women as well (37). The value of fulvestrant for premenopausal women and its value in the adjuvant setting remain to be further established.

LHRH agonists

Traditionally, ovarian ablation had been accomplished irreversibly via irradiation or surgery, but luteinizing hormone-releasing hormone (LHRH) agonists have emerged as reliable and reversible agents for this purpose. LHRH agonists act by desensitization of the hypothalamus-pituitary-ovarian axis. It has been demonstrated, that combination treatment of an LHRH agonist with tamoxifen is superior to treatment with an LHRH agonist alone in premenopausal women with advanced breast cancer (38).

AIs

In postmenopausal women, estrogens are biosynthesized from circulating androgens by the enzyme aromatase. Aromatase inhibitors anastrozole, letrozole, and exemestane have shown to be superior to tamoxifen in the first-line treatment of advanced disease (39-41). Although less severe than tamoxifen, aromatase inhibitors also show side-effects, including bone calcium loss (dependent on the type of AI) and musculoskeletal effects (42). As aromatase inhibitors are ineffective in premenopausal women with an intact hypothalamus-pituitary-ovarian axis, ovarian ablation and tamoxifen (combined with LHRH agonists or not), remain the only proven endocrine interventions for the treatment of these women.

MECHANISMS OF TAMOXIFEN-THERAPY FAILURE

Although tamoxifen has proven to be effective in treatment of breast cancer for many years now, resistance remains a common feature that limits the success of this type of therapy. It is known that not all patients who have ERα-positive tumors respond to tamoxifen therapy (intrinsic resistance) and even in patients who are initially responsive, the disease ultimately progresses (acquired resistance). Several recent review articles have discussed the mechanisms of tamoxifen resistance (43-46) and those are briefly summarized here.

Pharmacologic mechanisms

A common mechanism of drug resistance is a decreased intracellular drug concentration due to an altered in- or efflux, often mediated by membrane pumps. Such a mechanism has also been suggested to be involved in tamoxifen resistance, although the precise cause remains unclear (45 and references therein).

In the body, tamoxifen can be metabolized to *N*-desmethyltamoxifen, and the active forms 4-hydroxy-tamoxifen and 4-hydroxy-*N*-desmethyltamoxifen (endoxifen). These metabolites have different affinities for the ER and different plasma concentrations and thus influence the efficacy of tamoxifen. Besides CYP3A4, the enzyme CYP2D6 is responsible for the conversion of the inactive metabolites to the active metabolites. It has been shown that genetic variability in CYP2D6 may affect treatment outcome in patients receiving tamoxifen therapy (47).

Altered ER expression or function

Since the effects of tamoxifen are primarily mediated through ERα, and this gene has a prominent role in predicting the response to tamoxifen therapy, loss of ERα could confer resistance. Indeed, lack of expression of ERα is the main mechanism of intrinsic resis-

tance. However, it has been reported that patients with acquired resistance to tamoxifen usually do not lose expression of ERα. Loss has been demonstrated in 17-28% of these patients (48, 49). In fact, up to 20% of tamoxifen-resistant patients respond to second-line therapy with aromatase inhibitors or fulvestrant suggesting that their tumors still express functional ERα (50, 51). Mutations in or specific variants of ERα might also effect the response to tamoxifen (52-54), but published studies have shown that these are rare in breast-cancer patients and are unlikely to contribute significantly to tamoxifen resistance (55, 56). In addition, no conclusions on whether altered expression of ERβ may play a role in tamoxifen resistance can be drawn at this point.

Alterations in co-regulatory proteins

The transcriptional regulatory activity of ER is mainly mediated by the formation of complexes with co-activator or co-repressor proteins. Usually co-activators bind the ER when it is bound by estrogen, enhancing target gene transcription. When an antagonist such as tamoxifen is bound to ER, typically co-repressors are recruited which results in repression of target gene transcription. Under specific conditions, like high ERBB2 activity, a tamoxifen-ER complex may also recruit co-activator proteins, causing agonistic effects. Altered expression of co-regulators may therefore play a role in tamoxifen resistance (reviewed in 12, 57). It has been demonstrated *in vitro* that the co-activator proteins AIB1, PGC-1β, and SRC1 enhance the agonistic activity of tamoxifen (58, 59). In patients receiving adjuvant tamoxifen therapy, high levels of AIB1 alone or in combination with high levels of ERBB2 are associated with shorter disease-free survival in patients (60). These findings support a role for overexpression of co-activators in tamoxifen resistance. However, high levels of SRC1 were associated with favorable response to tamoxifen in patients (61), which does not fit with this hypothesis. The presence of other factors, like ERBB2, might play a role in this outcome (57). In addition, two studies showed that low levels of the co-repressor protein NCOR1 predict poor response to tamoxifen (62, 63). These results support the possibility that reductions in co-repressor activity may also contribute to tamoxifen resistance.

Modification of growth-factor signaling

Tamoxifen resistance may also be explained by altered expression and/or modification of several growth factor receptors and downstream signaling molecules. Resistant tumors often show activation or elevated levels of tyrosine kinase receptors such as the insulin-like growth factor 1 receptor (IGF-1R), the epidermal growth factor receptor (EGFR), and ERBB2. Activity of molecules that function downstream of these receptors like mitogen-activated protein kinase 1 and 3 (MAPK1 and 3, also known as ERK2 and ERK1), MAPK 14 (p38), AKT, and p21-activated kinase-1 (Pak1) may also be associated with resistance (44, 64). It is suggested that these growth factor receptors and their

downstream components mediate tamoxifen resistance via crosstalk with the ER as well as through independent pathways.

Despite tremendous advances in understanding growth factor-receptor and estrogen-receptor signaling, underlying mechanisms for general tamoxifen-therapy failure still have to be elucidated. Although alternative treatment strategies using modern SERMs and AIs are very promising, these therapies eventually fail as well. Thus it remains important to gain insights into the mechanisms underlying endocrine-therapy failure, because it will help us to develop new therapeutic strategies and to overcome endocrine resistance in breast-cancer patients.

EXPERIMENTAL STRATEGIES TO UNRAVEL TAMOXIFEN-THERAPY RESISTANCE

Non-functional profiling strategies

High-throughput molecular technologies provide insight into the DNA, RNA, and protein levels of complex biological samples. RNA expression profiling techniques, allowing simultaneous analysis of up to tens of thousands of genes, include PCR-based differential display (65), sequencing based serial analysis of gene expression (SAGE) (66), and hybridization-based DNA microarrays. In the last decades, various studies have been performed for identifying biological factors that predict the success of tamoxifen treatment (67). Paik *et al*. performed a reverse-transcriptase polymerase chain reaction (RT-PCR) assay on 250 prospectively selected genes generating a 21-gene profile that predicts the recurrence of tamoxifen-treated, node-negative breast cancer (68). Furthermore, two studies using microarray technology for generating a gene signature predictive for response to tamoxifen have been reported (69, 70). Ma and colleagues developed a two-gene signature, HOXB13/IL17BR, predictive for disease-free survival from 60 patients treated with adjuvant tamoxifen. From 46 tumors, Jansen *et al*. identified 81 genes that predicted for response to the therapy. However, a gene signature classifier that is good at prediction may not necessarily contain the genes that are functionally responsible for resistance. Functional proof has to be provided through transfection or functional knockdown using siRNAs.

Functional profiling strategies

For identifying genes causing tamoxifen resistance, several functional genetic screens were applied, namely transfection of candidate genes, cDNA library transfection, retrovirus insertion mutagenesis, and retroviral transduction of cDNA libraries. Transfection of candidate genes included the selection of several candidate genes from array data. Subsequently, the genes were introduced into estrogen-dependent breast-cancer cells

and tested for their altered hormone dependency (71). Toi *et al*. randomly searched for genes involved in tamoxifen resistance by transfecting cDNA libraries into tamoxifen-sensitive MCF-7 cells, though with limited success. One tamoxifen-resistant cell line was isolated, but involvement of the integrated cDNA was never established (72).

Retrovirus insertion mutagenesis and retroviral transduction of cDNA libraries were more successful approaches for finding genes causing tamoxifen resistance. In retrovirus-mediated insertion mutagenesis retroviruses randomly integrate into the genome of target breast cancer cells as part of their life cycle (73). As a result, they cause single genetic modifications which may enhance or disturb local gene expression, leading to an altered and selectable phenotype (Figure 1.3). Furthermore, the integrated viral

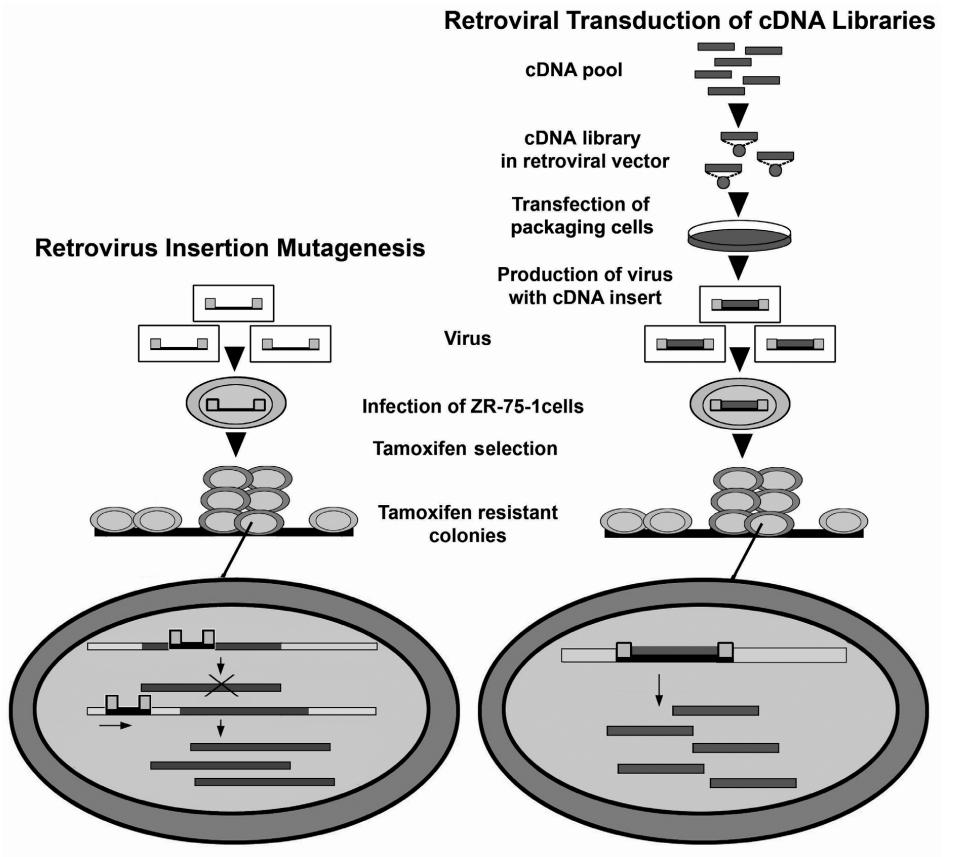

Figure 1.3 Retrovirus insertion mutagenesis and retroviral transduction of cDNA libraries have shown to be successful techniques for identifying genes responsible for tamoxifen resistance *in vitro*. In the first, retroviruses randomly integrate into the genome of target ZR-75-1 breast cancer cells as part of their life cycle. This results in alteration of local gene expression which may facilitate cell proliferation in the presence of tamoxifen. In the latter, packaging cells are transfected with cDNA libraries, resulting in production of replication-defective virus containing cDNA inserts. Subsequent infection of target cells causes cDNA overexpression in the cells and may facilitate cell proliferation in the presence of tamoxifen.

genome may be used as a tag to isolate the genes in question. Application of insertion mutagenesis in ZR-75-1 human breast cancer cells, revealed twelve common viral integration sites (cVISs) promoting tamoxifen-resistant cell proliferation (74-77 and manuscript in preparation).

Of Breast Cancer Antiestrogen Resistance (BCAR) genes 1 (p130Cas) and 3, two genes located within two cVIS loci, overexpression was shown to confer tamoxifen resistance. The molecular mechanism by which elevated expression of BCAR1 promotes proliferation still has to be elucidated. It is known to be independent from the ER and EGFR pathways (78) and may be involved in enhancing downstream signaling. BCAR3 induces tamoxifen resistance by enhancing PI3K-mediated Rac1 activation (79). Furthermore, BCAR3 associates with BCAR1 and increases cell migration, suggesting that BCAR3-mediated tamoxifen resistance is the result of altered adhesion-related signaling (80). In breast cancer patients, high levels of BCAR1 are associated with poor prognosis and predict a poor response to tamoxifen treatment in case of recurrent disease (81-84).

Although insertion mutagenesis was shown to be successful, the identification of the genes responsible for the tamoxifen-resistant phenotype appeared to be very labor-intensive. To overcome this technical limitation, a more rapid screening technique using retroviral transduction of cDNA libraries was used. With this technique, cells are infected with replication-defective retroviruses containing cDNAs (or siRNAs) derived from various tissues (Figure 1.3). Genes responsible for an altered selectable phenotype can easily be identified by PCR using virus-specific primers (85). Several genes that may be key players in the tamoxifen resistance of human breast cancer cells were identified using this approach (86). Recently, functional screens using RNAi have also resulted in the identification of genes involved in resistance to tamoxifen (87) and other treatment modalities (88-90).

AIMS AND OUTLINE OF THE THESIS

Although women with breast cancer have successfully been treated with tamoxifen during the past decades, resistance to this therapy remains an important issue. It is known that many different molecular mechanisms may account for tamoxifen resistance and identification of the genes involved may help improving diagnostics and individualizing breast-cancer treatment. The aim of this thesis is identifying genes involved in tamoxifen resistance. In chapter 2 a rapid screening strategy is described, revealing several causative genes. In chapter 3 the predictive value of FGF17, one of the causative genes, and the receptors *FGFR1, 2, 3* and *4* were assessed for the type of response to tamoxifen treatment and the duration of progression-free survival in patients with recurrent breast cancer. Combination of the results of a microarray study for generating a gene signature

predictive for response to tamoxifen treatment and the results of a functional screen for causative genes described in chapter 2, revealed two overlapping genes, TSC22D1 and PSAP. The association of these genes with the duration of progression-free survival in patients with recurrent breast cancer is described in chapter 4. Chapter 5 describes further characterization of BCAR4, a novel gene involved in tamoxifen resistance as was reported in chapter 2. Chapter 6 summarizes the major findings of the studies described in this thesis and discusses the future perspectives.

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CHAPTER 2

FUNCTIONAL SCREEN FOR GENES RESPONSIBLE FOR TAMOXIFEN RESISTANCE IN HUMAN BREAST CANCER CELLS

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ABSTRACT

Antiestrogens, such as tamoxifen, are widely used for endocrine treatment of estrogen receptor-positive breast cancer. However, as breast cancer progresses, development of tamoxifen resistance is inevitable. The mechanisms underlying this resistance are not well understood. To identify genes involved in tamoxifen resistance, we have developed a rapid screening method. To alter the tamoxifen-sensitive phenotype of human ZR-75-1 breast cancer cells into a tamoxifen-resistant phenotype, the cells were infected with retroviral cDNA libraries derived from human placenta, human brain, and mouse embryo. Subsequently, the cells were selected for proliferation in the presence of 4-hydroxy-tamoxifen (OH-TAM) and integrated cDNAs were identified by sequence similarity searches. From 155 OH-TAM-resistant cell colonies, a total of 25 candidate genes were isolated. Seven of these genes were identified in multiple cell colonies and thus cause antiestrogen resistance. The epidermal growth factor receptor, platelet-derived growth factor receptor-α, platelet-derived growth factor receptor-β, colony-stimulating factor 1 receptor, neuregulin 1, and fibroblast growth factor 17 that we have identified, have been described as key regulators in the mitogen-activated protein kinase pathway. Therefore, this pathway could be a valuable target in the treatment of patients with breast cancer resistant to endocrine treatment. In addition, the putative gene LOC400500, predicted by *in silico* analysis, was identified. We showed that ectopic expression of this gene, designated as breast cancer antiestrogen resistance 4 (BCAR4), caused OH-TAM resistance and anchorage-independent cell growth in ZR-75-1 cells and that the intact open reading frame was required for its function. We conclude that retroviral transfer of cDNA libraries into human breast cancer cells is an efficient method for identifying genes involved in tamoxifen resistance.

INTRODUCTION

Tamoxifen is the most extensively used antiestrogen in the treatment of breast cancer. Patients with estrogen receptor (ER)-positive breast tumors may initially benefit from this treatment, but almost all responding patients acquire resistance to the action of tamoxifen over time and the disease progresses. Several mechanisms for this phenomenon have been suggested, including alteration of the availability or metabolism of tamoxifen, alterations in the function of the ER and in the ER signaling cascade, and the altered expression of different genes (reviewed by 1, 2). However, in the majority of patients, the mechanisms causing tamoxifen-resistant proliferation remain unexplained. Insight into these processes is essential for the development of improved treatment strategies and may be obtained by the application of genome-wide functional screens.

Random transfection of cDNA libraries was previously used to identify the specific genes involved in progression to antiestrogen resistance of human breast cancer cells, but this had only limited success (3). On the other hand, our group has successfully identified such genes using retroviral insertion mutagenesis in functional screens (4). Although insertion mutagenesis has shown itself to be a very powerful tool in the identification of genes involved in mouse tumorigenesis, in our experiments the identification of the genes responsible for antiestrogen-resistant proliferation has proved to be very labor intensive (5-7).

To bypass this technical limitation, we have developed a rapid screening strategy for identifying genes that cause tamoxifen-resistant cell proliferation. In this study, we used replication-defective retroviruses to express cDNAs, a strategy that has previously been used for various experimental purposes (8-10). We applied this methodology to estrogen-dependent human ZR-75-1 breast cancer cells and here report the identification of several genes that may be key players in the resistance of human breast cancer cells to antiestrogenic drugs.

RESULTS

Functional screens for tamoxifen resistance

ZR-75-1 human breast cancer cells were infected with retroviral cDNA libraries derived from either human placenta, human brain, or mouse embryo (Figure 2.1). After infection, the cells were selected for growth in the presence of 4-hydroxy-tamoxifen (OH-TAM). The first resistant colonies were observed 3 weeks after retroviral transduction, and all colonies were picked within 6 weeks after start of the experiments. Three independent cell cultures (a total of 1.2 x 10^7 cells) were infected with the human placenta cDNA library, and 80 tamoxifen-resistant cell colonies were isolated. In addition, 30 cell

colonies were isolated from three flasks with independent cell cultures (10^7 cells each) infected with the mouse embryo cDNA library. To prevent the isolation of many identical cell colonies due to reseeding of proliferating cells, 32 individual 25cm^2 flasks with approximately 10^6 cells each (a total of 3.2 x 10^7 cells) were infected with viral particles derived from a human brain library. In total, 45 cell colonies were isolated from the cell cultures infected with this library. In control experiments, ZR-75-1 cells mock infected or infected with pLIB-EGFP virus failed to produce proliferating colonies in the presence of OH-TAM within 6 weeks. In total, 7.4 x 10^7 cells were transduced with viral particles of three different cDNA libraries, resulting in 155 resistant cell colonies. The estimated infection frequency was approximately 10%; thus, the frequency of cell colonies was at least a 2,000-fold higher than that of spontaneous antiestrogen-resistant cell colonies in control cultures (below 10^{-8}) (4).

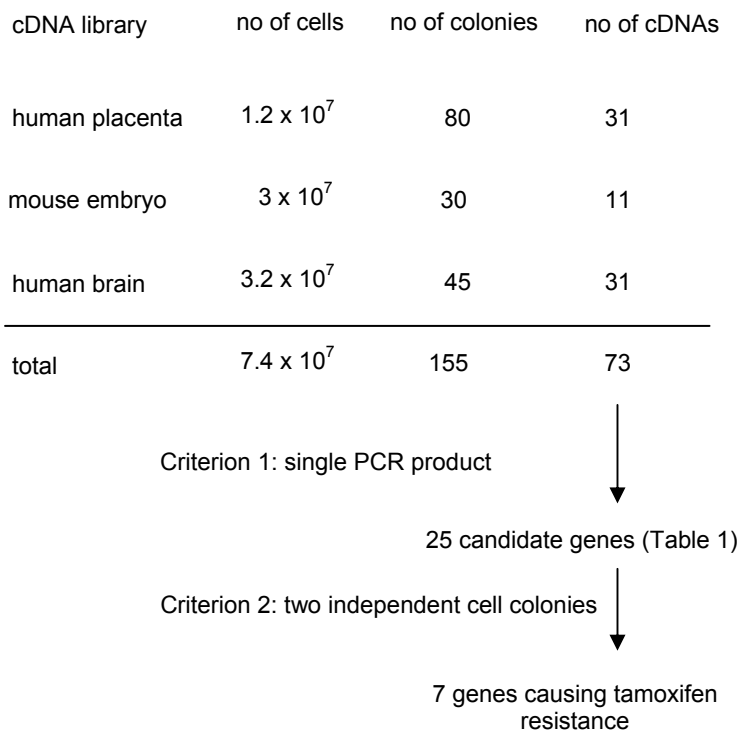

Figure 2.1 Study outline: Functional screen for tamoxifen resistance.
ZR-75-1 cells were transduced with retroviral cDNA libraries derived from human placenta (3 independently infected cell cultures), mouse embryo (3 independently infected cell cultures) and human brain (32 independently infected cell cultures). A total of 73 different genes were identified. In 25 colonies only one integrated cDNA was observed, suggesting a causative role in tamoxifen resistance for the genes identified (Table 1). Seven of these genes were isolated from at least two independently derived cell colonies, complying with our second criterion.

Genes recovered from screens for tamoxifen resistance

Genomic DNA was isolated from resistant cell colonies, and integrated cDNAs were recovered using PCR assays. These assays showed that the majority of the tamoxifen-resistant cell colonies contained only one retroviral insert (range 1-6; median 1). Approximately one third of the isolated colonies contained multiple retroviral integrations.

In one of the OH-TAM-resistant cell lines obtained after infection with the placenta cDNA library, PCR analysis failed to detect an integrated cDNA. Southern blot analysis showed that this particular cell line contained an integrated retrovirus with only one retroviral long terminal repeat, likely the result of a truncation event during integration of the retrovirus in the host genome. The integrated cDNA in this cell line was successfully isolated using an inverse DNA amplification technique (11, 12). This phenomenon indicates that not every integrated cDNA may be detected by PCR analysis using primers located adjacent to the inserted cDNAs. Therefore, truncated retroviruses may lead to misconceived conclusions for some of the genes identified in a screen. In addition, we have shown previously that tamoxifen-resistant cell colonies may result from insertion mutagenesis of a retrovirus independent of its cDNA insert at a frequency of one colony per approximately 2 million cells (4). To ascertain that an inserted cDNA was actually responsible for tamoxifen resistance and to exclude wrong assignments as a consequence of virus truncation or insertion mutagenesis events, we applied the following criteria. First, each candidate cDNA represented a single product following 35 cycles of PCR with genomic DNA from at least one tamoxifen-resistant cell colony. Second, a particular cDNA should be recovered from at least two independently derived cell colonies. This independence was established by differences in length of the 5'-noncoding region of the inserted cDNAs or if cell colonies originated from infections of independent cell cultures. Analyses of the cDNAs isolated from the resistant colonies revealed a total of 73 different genes (Figure 2.1). Twenty-five genes (listed in Table 2.1) complied with the first criterion and are considered candidate genes. Seven of these genes also complied with the second criterion and thus caused tamoxifen resistance in ZR-75-1 cells. We fully sequenced the cDNA inserts of two independent colonies of each of these seven genes and confirmed the presence of cDNAs containing the complete open reading frame lacking mutations.

Among the seven genes causing tamoxifen resistance, four different receptor tyrosine kinases (RTK) were present: epidermal growth factor receptor (EGFR), colony-stimulating factor 1 receptor (CSF1R), mouse platelet-derived growth factor (PDGF) receptor-α (Pdgfra), and both mouse and human PDGF receptor-β (Pdgfrb and PDGFRB). Four cell colonies with integrated EGFR cDNAs were isolated from one cell culture infected with the human placenta library, as were two CSF1R cDNAs. Lenght differences at the 5'-end detected by sequence analysis confirmed their independent origin. Six of seven Pdgfra cDNAs were recovered from two independent cell cultures infected with the mouse

embryo library. PDGFRB was identified in 12 colonies retrieved from one cell culture infected with the placenta library. At least two cell colonies had an independent origin, because the inserted cDNAs differed at the 5'-end. Seventeen Pdgfrb cDNAs were

Table 2.1 Summary of the genes identified in the screen*

Gene	NCBI UniGene	No of total isolates†	No of independent isolates‡	Library#	Function§
LOC400500	Hs.24611	52	26	hp	unknown
Pdgfrb	Mm.4146	17	14	me	RTK‖
PDGFRB	Hs.509067	12	2	hp	RTK
FGF17	Hs.248192	12	8	hb	ligand of RTK
NRG1**	Hs.453951	8	6	hb	ligand of RTK
Nrg1**	Mm.153432	4	2	me	ligand of RTK
Pdgfra	Mm.221403	7	6	me	RTK
EGFR	Hs.488293	4	2	hp	RTK
CSF1R	Hs.483829	2	2	hp	RTK
Psap	Mm.277498	2	1	me	enzyme activator
ASMTL	Hs.533514	2	1	hp	unknown
ALK	Hs.196534	2	1	hb	RTK
APLP1	Hs.74565	1	1	hb	protein binding
APP	Hs.434980	1	1	hb	signaling
CBFA2T3	Hs.513811	1	1	hb	transcription
ERBB2	Hs.446352	1	1	hp	RTK
GFAP	Hs.514227	1	1	hb	Intermediate fillament
GFI1B	Hs.118539	1	1	hp	zinc finger protein
NEDD9	Hs.37982	1	1	hp	signaling
HRAS	Hs.37003	1	1	hp	signaling
KIAA0513	Hs.301658	1	1	hb	unknown
L1CAM	Hs.522818	1	1	hb	cell adhesion
CLDN23	Hs.183617	1	1	hp	cell adhesion
MDC1	Hs.433653	1	1	hb	DNA repair
PB1	Hs.189920	1	1	hb	DNA binding
PTMA	Hs.459927	1	1	hp	transcription
STX1A	Hs.488683	1	1	hb	protein binding

* Inserted cDNAs were listed when they were the only product after 35 PCR cycles in the analysis of at least one cell colony. Seven of these cDNAs were found at least twice as independent event.

† Number of cell colonies identified with the respective gene.

‡ Number of independent cDNAs found using a particular cDNA library.

hp: human-placenta library, hb: human-brain library, me: mouse-embryo library.

§ Function was derived from the NCBI/Gene database.

‖ RTK: receptor tyrosine kinase.

** only transcript variant SMDF was identified.

isolated from resistant colonies retrieved from cell cultures infected with the mouse embryo library. Fourteen of these differed at the 5'-end, or were recovered from autonomous cell cultures and have thus arisen independently. In addition to the RTK genes, we identified mouse and human neuregulin1 (Nrg1 and NRG1) and human fibroblast growth factor (FGF) 17. The Nrg1 gene was isolated four times from two independent cell cultures infected with the mouse embryo library, and the human NRG1 was isolated eight times from six cell cultures independently infected with the brain library. All cell colonies contained the NRG1 sensory and motor neuron-derived factor isoform (SMDF). FGF17 was identified in 12 colonies isolated from eight independent cell cultures infected with the human brain library.

LOC400500 (assigned as breast cancer antiestrogen resistance 4 (BCAR4), see discussion), a putative gene predicted by *in silico* analysis of the human genome (Hs.24611, accession no. XM_378564), was the cDNA most frequently found. Based on sequence and Southern blotting analysis of PCR products, we recovered LOC400500 52 times only in the different cell cultures infected with the human placenta library. At least 26 of these cDNAs were proven to be of independent origin. Sequence similarity analyses showed that the cDNAs had variable 5'-ends. However, all cDNAs contained an open reading frame encoding a 121-amino acid polypeptide identical to the open reading frame predicted for LOC400500 gene.

Ectopic Expression of LOC400500/BCAR4 Induces Antiestrogen Resistance and Anchorage-Independent Transformation

Additional experiments were done to confirm that the putative gene LOC400500 is able to induce tamoxifen-resistant proliferation in human ZR-75-1 breast cancer cells. ZR-75-1 cells were infected with retroviruses containing either an LZRS-IRES-Neo/LOC400500 expression construct or the LZRS-IRES-Neo expression vector without insert, designated as ZR/BCAR4 and ZR/LZRS, respectively. After infection, cells were selected with G418 in 17β-estradiol (E_2)-containing medium. Proliferation of the different cell pools, originating from approximately 10^5 colonies each, was similar in the presence of E_2-supplemented medium (Figure 2.2A). We next determined the capacity of the transduced cells to proliferate in the presence of OH-TAM. In Figure 2.2B proliferation curves over a 21-day culture period of these cells are presented. These curves show the dominant role of LOC400500/BCAR4 in tamoxifen-resistant proliferation of ZR-75-1 cells.

To establish whether the protein predicted for LOC400500/BCAR4 is responsible for tamoxifen resistance, a frameshift mutation was introduced using site-directed mutagenesis. This resulted in the addition of 20 heterologous amino acids after amino acid position 5 before reaching a stop codon. ZR-75-1 cell cultures were infected in duplicate with virus containing this frameshift construct (ZR/BCAR4-fs). Parallel infections were done with BCAR4 or LZRS virus to generate control cultures (ZR/BCAR4 and ZR/LZRS,

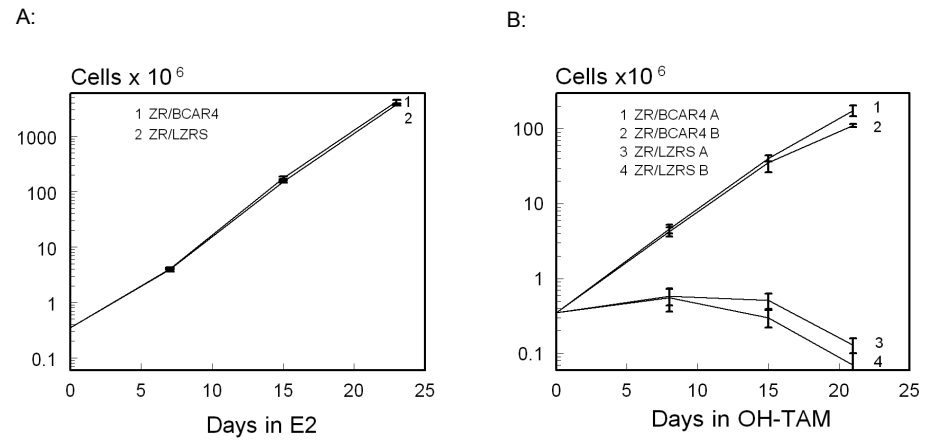

Figure 2.2 LOC400500/BCAR4 induces antiestrogen resistant proliferation.

Independent pools of ZR-75-1 cells, infected with virus from the expression vector without cDNA insert (ZR/LZRS) or containing LOC400500/BCAR4 (ZR/BCAR4), were plated in medium containing E_2 or OH-TAM in triplicate. At the timepoints indicated, the cells were counted and replated at the initial density. Cumulative cell numbers ± 95% confidence interval are presented over a 21 day culture period. (A) proliferation curves of the cells in medium containing E_2. (B) proliferation curves of the cells in duplicate in medium containing OH-TAM.

respectively). Two days after infection, 5% of the cells were plated in medium containing OH-TAM. In the ZR/BCAR4 cultures, proliferating cell colonies (average of 4,100 per flask) were observed within 3 weeks, whereas no proliferating cell colonies could be detected in ZR/BCAR4-fs cultures and in ZR/LZRS control cultures up to 5 weeks. Another 5% of the cells were plated in medium containing E_2 plus G418, and successful infection was shown by the presence of G418-resistant colonies in ZR/BCAR4, ZR/BCAR4-fs, and ZR/LZRS cell cultures (average of 5,000, 3,950, and 4,150 colonies per flask, respectively). These data argue for a direct role of the protein instead of the RNA.

A soft agar assay was used to determine anchorage-independent transformation of ZR/BCAR4 cells *in vitro*. Parental ZR-75-1 cells are only capable of anchorage-independent proliferation in the presence of estradiol. In the absence of estradiol colony formation is almost fully abrogated. ZR-75-1 vector control and ZR/BCAR4 cells (1×10^5) were plated in bovine calf serum-supplemented soft agar in duplicate. In ZR-BCAR4 cell cultures, colonies appeared within 10 days and were counted after 5 weeks. Large numbers of colonies were observed for ZR/BCAR4 cells (16,800 per 1×10^5) compared with ZR-75-1 vector control cells (45 per 1×10^5; Figure 2.3).

DISCUSSION

In this study, we identified seven genes involved in antiestrogen-resistant proliferation. Our breast cancer cell model for antiestrogen resistance has proven to be almost background-free, allowing the identification of genes involved in tamoxifen resistance

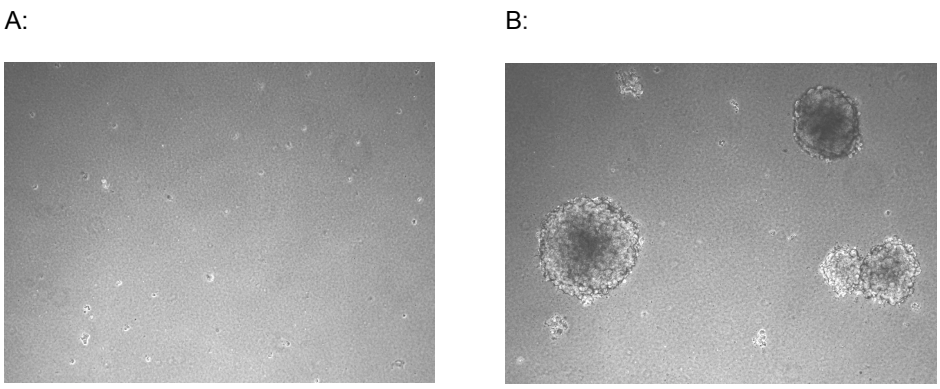

Figure 2.3 LOC400500/BCAR4 induces anchorage-independent transformation.
A total of 1 x 10^5 ZR-75-1 vector control cells (ZR/LZRS) and 1 x 10^5 ZR-75-1 cells containing LOC400500/BCAR4 (ZR/BCAR4) were plated in BCS-supplemented soft agar in duplicate. Representative light-microscopy photographs of flasks with ZR/LZRS (A) or ZR/BCAR4 (B) cells after five weeks are shown.

using insertion mutagenesis (4). Random insertion of retroviruses in the cellular genome causes local disturbance of the genome structure (13) and may affect expression of adjacent genes (14, 15). Infection of ZR- 75-1 cells resulted in the identification of seven BCAR genes and an additional seven common viral integration sites (5-7, and in preparation). The frequency of retrovirally induced antiestrogen-resistant colonies was approximately 5 x 10^{-7} (4), and the probability of activating one of the BCAR genes most frequently found in our study was at least 10-fold lower. Our current experiments with retroviral transduction of cDNA libraries derived from human placenta, human brain, and mouse embryo proved to be 40 times more efficient in inducing tamoxifen resistance than insertion mutagenesis. Furthermore, identifying the genes responsible was less time consuming. On the other hand, the nature of the cDNAs identified in the screens is strongly dependent on the cDNA library used, whereas insertion mutagenesis is an almost random process. The contribution of insertion mutagenesis events in this study can be ignored in view of its very low frequency (5 x 10^{-7}) and the application of our stringent selection criteria, as detailed in the study outline and Table 1. Hence, this strategy obviates the need for a second round of selection involving replication-competent amphotropic viruses or time-consuming construction of suitable expression vectors.

Of the 73 genes identified in our current screens, 7 fulfilled both selection criteria and are thus considered to be responsible for antiestrogen resistance. Two of these genes (EGFR and LOC400500/BCAR4) have been confirmed to cause tamoxifen resistance in ZR-75-1 cells by gene transfer experiments (16, and this article). Four of these genes are expressed at extremely low levels (EGFR) or are undetectable (PDGFRA, PDGFRB, and LOC400500/BCAR4) in the parental ZR-75-1 cells (17, and unpublished results). Thus, the infected cells acquired a novel function essential for tamoxifen-resistant proliferation.

Eighteen of the 73 genes complied only with the first criterion (Figure 2.1; Table 2.1) and are considered to be interesting candidates. Formal prove of their role in tamoxifen resistance may be obtained in additional cDNA library transduction experiments or by individual gene transfer experiments.

The majority of the genes identified in this study are involved in RTK signaling. We identified four full length RTKs: mouse Pdgfra, both human and mouse PDGFRB, human CSF1R and human EGFR, which have previously been classified into two subfamilies on the basis of common structural features (18).

PDGFRA, PDGFRB and CSF1R are members of the PDGFR RTK subfamily, which also encompasses KIT and the FGF receptors. Previous studies have shown the presence of PDGFRB mRNA in breast cancer cells (19). Aberrant expression of CSF1R has also been documented in a variety of malignancies, including breast cancer (reviewed by 20). In mice bearing human breast cancer xenografts and treated with small interfering RNAs directed specifically against the CSF1R mRNA, tumor growth was suppressed by 40 to 50% and mouse survival increased (21).

EGFR is part of an RTK subfamily, including ERBB2, which is also among our list of candidate genes (Table 2.1), and ERBB3, and ERBB4. EGFR has been reported previously to play an important role in normal development, differentiation, and cell proliferation (reviewed by 22). High levels of EGFR promote antiestrogen-resistant proliferation in breast cancer cells and are associated with failure of tamoxifen treatment (16, 23). However, because expression of EGFR is inversely related to expression of ERα in breast cancers (24, 25), it is unlikely that EGFR plays a dominant role in antiestrogen resistance in ER-positive breast cancers.

We also identified the EGF-like ligand NRG1, which interacts with ERBB3 and ERBB4 receptors, leading to formation of ERBB homodimers or heterodimers (often including ERBB2). In our screens using both a human brain and a mouse embryo cDNA library we identified the NRG1 sensory and motor neuron-derived factor isoform, which shares only the EGF-like domain with other NRG1 isoforms (26). High expression levels of NRG1 have been shown to cause progression of MCF-7 breast cancer cells to an estrogen-independent and antiestrogen-resistant state, which was later mimicked in a transgenic mice model (27).

FGF17, a ligand for the FGF receptor subfamily, was identified as well. Among known FGF family members, the FGF17 protein is most similar to FGF8 (28). Together with FGF8 and FGF18, it forms a subfamily, having similar gene structures, overlapping patterns of expression, and receptor-binding specificities (29). Overexpression of FGF8 and FGF17 in NIH-3T3 cells results in a transforming and tumorigenic phenotype (30, 31). Furthermore, FGF8 expression is significantly higher in breast cancer and in prostate cancer than in nonmalignant tissues (32, 33). Although no data are available for FGF17 expression in breast cancer yet, Heer *et al.* (34) reported a significant increase in FGF17 mRNA

expression in high-grade prostate cancers compared with benign prostatic hypertrophic tissues.

In addition to these known genes, we identified LOC400500, a putative gene positioned at human chromosome 16p13.1. It was recovered from 65% of the tamoxifen-resistant cell colonies obtained after transduction with a human placenta cDNA library. The gene has been designated as BCAR4. Sequence similarity analyses showed that all BCAR4 cDNAs contained the same open reading frame of 121 amino acids. The amino acid sequence shows no homology with other proteins yet nor typical conserved domains. The presence of a putative signal peptide sequence (www.ebi.ac.uk/InterProScan/) suggests that BCAR4 is transported to the membrane. The presence of two putative transmembrane regions further supports this hypothesis. The only homologues of BCAR4 reported until now were found in chimpanzee (100% identical) and rhesus monkey (92% identical). Thus, this gene may have emerged during the primate evolution. Here, we showed that this novel identified gene causes antiestrogen resistance in ZR-75-1 cells. Furthermore, loss of antiestrogen resistance due to a frameshift mutation strongly suggests that expression of the small protein is responsible for this resistance. We also showed anchorage-independent transformation of BCAR4 cells in a soft agar assay. From serial analysis of gene expression data (http//bioinfo.amc.uva.nl/HTMseq/) and EST profiles (www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Hs.24611), it is clear that BCAR4 is expressed in placenta and embryo. In addition, analysis of our expression data (35) and published array data (36, 37) indicates that BCAR4 expression is present in approximately 10% of breast cancers.

In our future studies, we will establish which genes identified *in vitro* are important in progression of breast cancer in patients. We have already documented that genes identified in a screen for tamoxifen resistance can have clinical relevance (38, 39). The results from our planned studies will enable us to select particular genes for further study and to identify key regulators susceptible to targeted therapy.

In conclusion, retroviral transfer of cDNA libraries to human breast cancer cells is an efficient method for identifying genes involved in antiestrogen resistance *in vitro*. Six of seven genes that were identified in multiple resistant cell colonies are part of the mitogen-activated protein kinase pathway. This suggests that mitogen-activated protein kinase signaling may play an important role in tamoxifen resistance in human breast cancer. With the recent development of new tyrosine kinase inhibitors and antireceptor monoclonal antibodies, other genes involved in RTK signaling besides EGFR and ERBB2 may represent valuable targets for therapy of tamoxifen-resistant breast cancer (40).

MATERIALS AND METHODS

Cell lines and culture conditions

ZR-75-1 cells (41) were cultured in RPMI 1640 (Invitrogen, Paisley, United Kingdom) supplemented with 10% heat-inactivated bovine calf serum (standard medium; Hyclone, Logan, UT) and supplemented with 1 nmol/L E_2 (Sigma-Aldrich, St. Louis, MO) as described previously (16).

Cell colonies resistant to OH-TAM (Sigma-Aldrich) were picked and expanded in RPMI 1640 supplemented with 10% bovine calf serum and 10% conditioned medium of CRIP cells (mouse fibroblast cell line) and 1 μmol/L OH-TAM (4).

Transduction of retroviral cDNA libraries into ZR-75-1 cells

Retroviral transduction experiments were done using retroviral cDNA expression libraries derived from human placenta, human brain, and mouse embryo (Clontech, Palo Alto, CA). Retroviral particles were produced by transient transfection of Phoenix-Ampho packaging cells (42) using FuGENE 6 (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Cell-free supernatants containing viral particles were harvested after 2 and 3 days and stored at -80°C. In total, 38 flasks with ZR-75-1 cells were incubated with pLIB-cDNA virus in the presence of 4 μg/mL polybrene (Sigma-Aldrich) and 1 nmol/L E_2 to stimulate proliferation and integration of the retroviruses. To determine the infection frequency with fluorescence-activated cell sorting or fluorescence microscope analysis, cells were infected with a mixture of viral supernatant containing pLIB-EGFP (Clontech) together with viral supernatant of the placenta cDNA library. Alternatively, 1% of pLIB-EGFP construct was mixed with the library cDNA plasmids before production of the viral particles. Three days after infection, the cells were trypsinized and resuspended. The cells were plated in 75 cm^2 flasks (4 x10^6 per flask) in medium containing 1 μmol/L OH-TAM. Within 6 weeks of plating, OH-TAM-resistant colonies were picked and transferred to 96-well or 48-well plates. Subsequently, the colonies were expanded for further characterization. Genomic DNA was isolated using NaCl extraction procedures as described by Miller *et al.* (43).

Analysis of proviral cDNA inserts

Integrated cDNAs were retrieved from genomic DNA by PCR using primers located in pLIB adjacent to the cDNA cloning site (44). PCR was done using the Expand High Fidelity PCR System (Roche) and the Expand Long Template PCR System (Roche) according to the manufacturer's instructions. PCR products containing multiple fragments were separated on and purified from 0.7% agarose gels using QIAquick gel extraction kits (Qiagen, Hilden, Germany). Sequence analysis was done on a LI-COR sequencer (LI-COR Inc., Lincoln, NE) using Thermo Sequenase DYEnamic direct cycle sequencing kits (Am-

ersham plc, Buckinghamshire, United Kiongdom) or on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) using ABI Prism BigDye Terminators v3.0 cycle sequencing kits (Applied Biosystems) according to the protocols of the manufacturers. The cDNAs were identified by sequence similarity searches using Basic Local Alignment Search Tools (www.ncbi.nlm.nih.gov/BLAST/).

Expression constructs and proliferation assays

A cDNA, containing the predicted coding region of LOC400500, isolated from one of the resistant cell lines, was cloned into the LZRS-IRES-Neo expression vector (45) of which viral particles were produced. ZR-75-1 cells were infected in duplicate with retroviral particles containing either the LOC400500 expression construct (ZR/BCAR4) or the LZRS-IRES-Neo expression vector without insert (ZR/LZRS). The cells were cultured in E_2-containing medium, and after 2 days, selection with G418 (Invitrogen) was started. G418-resistant cell colonies were pooled and expanded. Pools of ZR/BCAR4 and ZR/LZRS cells were harvested by trypsinization and counted with a Coulter Z1 cell counter (Coulter Electronics Ltd, Luton, United Kingdom). Subsequently, 3.5×10^5 cells were plated in triplicate in 25 cm^2 tissue culture flasks. Experimental medium containing 1 nmol/L E_2 or 1μmol/L OH-TAM was changed twice weekly. Cells were trypsinized, counted, and replated in fresh medium at the initial density once weekly.

A frameshift construct of LOC400500 was produced by inserting a thymine directly following the codon for amino acid 4. We did this site-specific mutagenesis with mutated PCR primers, and the resulting construct was sequence verified. ZR-75-1 cells were infected in duplicate with retroviruses containing the frame-shift construct (BCAR4-fs). ZR/BCAR4 and ZR/LZRS cells were used as control cultures. The cells were cultured in E_2-containing medium and trypsinized and resuspended after 2 days. Five percent of the cells were plated in medium containing 1 μmol/L OH-TAM. Colonies of proliferating cells were counted after 15 days. To establish whether the infection was successful, another 5% of the cells were plated in 25 cm^2 flasks in E_2-containing medium. After 2 days, G418 selection was started and colonies of proliferating cells were counted 6 days after start of selection.

To determine *in vitro* anchorage-independent transformation of ZR/BCAR4 cells, a soft agar assay was done. ZR/LZRS and ZR/BCAR4 cells (1×10^5) were plated in duplicate in 0.3% soft agar with 10% bovine calf serum in RPMI1640 culture medium on 0.6% base agar layers containing the same ingredients. Colonies were counted after 5 weeks.

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CHAPTER 3

FIBROBLAST GROWTH FACTOR RECEPTOR 4 PREDICTS FAILURE ON TAMOXIFEN THERAPY IN PATIENTS WITH RECURRENT BREAST CANCER

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ABSTRACT

Tamoxifen treatment of estrogen-dependent breast cancer ultimately loses its effectiveness due to the development of resistance. From a functional screen for identifying genes responsible for tamoxifen resistance in human ZR-75-1 breast cancer cells, fibroblast growth factor (FGF) 17 was recovered. The aim of this exploratory study was to assess the predictive value of FGF17 and the receptors FGFR1-4 for the type of response to tamoxifen treatment (clinical benefit) and the duration of progression free survival (PFS) in patients with recurrent breast cancer. Messenger RNA levels of FGF17 and FGFR1-4 were quantified by real-time reverse transcriptase PCR in 285 estrogen receptor-positive breast carcinomas with clinical follow-up. All patients had recurrent disease and were treated with tamoxifen as first-line systemic therapy for local or distant relapse. FGF17 and FGFR1-3 mRNA levels had no significant predictive value for this group of patients. However, high FGFR4 mRNA levels analyzed as a continuous log-transformed variable predicted poor clinical benefit (odds ratio = 1.22; $P = 0.009$) and shorter PFS (hazard ratio = 1.18; $P < 0.001$). In addition, in multivariable analysis, the predictive value of FGFR4 was independent from the traditional predictive factors. Our analyses show that FGFR4 may play a role in the biological response of the tumor to tamoxifen treatment. In addition, as altered expression of FGF17 causes tamoxifen resistance *in vitro*, the FGF-signaling pathway could be a valuable target in the treatment of breast cancer patients resistant to endocrine treatment.

INTRODUCTION

The fibroblast growth factors (FGFs) make up a large family of ligands that signal through cell-surface tyrosine kinase FGF receptors (FGFRs). In humans, 22 distinct FGFs and 4 FGFRs have been identified. All FGFs share important similarities, including significant sequence homologies at both the DNA and the protein levels (reviewed by 1). During embryonic development, FGF signaling has been associated with proliferation, migration and differentiation. In adults, FGFs are homeostatic factors that function in control of the nervous system, tissue repair, response to injury and tumor angiogenesis (reviewed by 1, 2).

The four FGFRs have an extracellular ligand-binding domain, a transmembrane domain and a split intracellular tyrosine kinase domain. The ligand-binding domain of the receptors is composed of three immunoglobin like domains I, II and III. FGFR1-3 all undergo alternative splicing using two alternative exons (IIIb and IIIc) to encode the carboxy-terminal half of immunoglobin domain III. These splice variants are expressed in a tissue-specific manner with exon IIIb variant expression restricted to epithelial lineages, and exon IIIc variant expression preferentially in mesenchymal lineages. Alternative splicing also contributes to the receptor binding specificity. Unlike FGFR1-3, FGFR4 is not alternatively spliced in this region (2, 3), but other variants have been reported (4).

FGF17 was identified in our functional screen as a gene causing tamoxifen resistance in ZR-75-1 human breast cancer cells (5). Among known FGF family members, the FGF17 protein shows the highest similarity with FGF8 (6). Together with FGF8 and FGF18 it forms a subfamily, having similar gene structures, overlapping patterns of expression and receptor binding specificities (1). Over-expression of FGF8 and FGF17 in NIH-3T3 cells results in a transforming and tumorigenic phenotype (7, 8). Furthermore, FGF8 expression has been demonstrated to be significantly higher in breast cancer than in non-malignant breast tissues (9). No data were available for FGF17 expression in breast cancer yet.

In order to investigate the association of FGF17 expression and expression of the receptors FGFR1-4 with tamoxifen treatment in breast cancer patients, a retrospective study was performed. Gene mRNA levels were measured by real time quantitative reverse transcriptase PCR (RT-PCR) in 285 estrogen receptor (ER)-positive frozen primary breast tumors from patients who developed recurrent disease that was treated with tamoxifen as first-line therapy. This report describes the association of FGF17 and the FGF receptors with first-line tamoxifen treatment in patients with recurrent breast cancer.

PATIENTS AND METHODS

Patients

The institutional Medical Ethical Committee approved our study design (MEC 02.953), which was carried out according to the Code of Conduct of the Federation of Medical Scientific Societies in The Netherlands (http://www.fmwv.nl/). Frozen tumor samples were originally submitted to our reference laboratory from regional hospitals for measurements of steroid hormone receptors and have been stored in our tumor bank at the Erasmus Medical Center (Rotterdam, The Netherlands). Guidelines for primary treatment were similar for all hospitals. All available frozen tumor specimens from female patients with breast cancer who entered the clinic during 1979–1995 and from whom detailed clinical follow-up was available (10, 11), were processed for mRNA analysis. Further inclusion criteria were: >100 mg frozen tissue available, invasive breast cancer, no previous other cancer (except basal cell skin cancer or early-stage cervical cancer stage Ia/Ib), no second primary breast tumor at first relapse, no adjuvant systemic hormonal treatment, >30% invasive tumor cell nuclei, and good RNA quality. Samples were rejected because of insufficient frozen tumor material (approximately 50%), too low percentage tumor cells (approximately 15%) and poor RNA quality (approximately 9%). A total of 285 primary, ER-positive breast tumor samples were included in this retrospective study. These patients were treated either with breast-conserving surgery (36%) or with modified mastectomy (64%). An axillary node dissection was performed in 93% of the patients (n = 268, 148 patients node positive). In contrast to current clinical practice, not all node-positive patients received adjuvant systemic therapy. This is due to the fact that tumors that were surgically removed from 1979 onwards were included in this study, and up to around 1990 it was not common clinical practice in The Netherlands to offer adjuvant systemic therapy to all node-positive patients. In total, 211 patients were postmenopausal (64% at primary surgery, 74% at start first-line therapy) and all had ER-positive tumors. ER status was determined by routine ligand-binding assays or enzyme immunoassays, and pathological examination was not performed centrally and reflects daily clinical practice in the various participating regional hospitals as described previously (12). None of the patients had received neoadjuvant therapy or were exposed to hormonal adjuvant treatment. Half of the patients received adjuvant radiotherapy and 52 patients were treated with adjuvant chemotherapy (19 patients anthracyclin-based (FAC/FEC) and 33 patients non-anthracyclin-based (CMF)). Thirty patients had metastasis at diagnosis or developed distant metastasis (including supraclavicular lymph node metastasis) within 1 month after primary surgery.

All patients, including 32 patients with a local recurrence, were treated for recurrent disease with first-line tamoxifen therapy (40 mg daily). Median time to treatment was 28 months. The median age of the patients at the time of primary surgery was 57 years

(ranges 26-89 years) and at the start of tamoxifen therapy for recurrent disease 61 years (ranges 29-90 years). Patients were routinely followed at the outpatient clinic, generally once every 3 weeks during the first 6 months, and in case of objective response approximately every 6 weeks later on (13). Skin metastases were assessed clinically by palpation and documented by photography; lymph node metastases were assessed by palpation and sometimes if necessary by ultrasound; lung metastases were routinely followed by X-thorax (once every 6-12 weeks), and by CT-thorax where applicable; liver metastases were always followed by CT of the liver, in general once every 12 weeks; brain metastases were assessed by magnetic resonance imaging if indicated; and bone metastases were followed by X-rays (every 6-12 weeks) and bone scan (every 6-12 months) as a standard, and by magnetic resonance imaging if indicated. Furthermore, plasma tumor marker levels (CA15.3 and/or CA125) were regularly measured. The type of response to tamoxifen therapy was recorded as defined by standard Unio Internationale Contra Cancrum criteria (14). In total 179 patients, with complete remission (i.e. complete disappearance of all metastases, n = 13), partial remission (i.e. at least 50% reduction, n = 39) or with stable disease longer than 6 months (n = 127), were classified as patients having clinical benefit (responders) as defined in the manual for clinical research and treatment in breast cancer of the European Organization for Research and Treatment of Cancer (15). Fifteen patients with stable disease shorter than or equal to 6 months and 91 patients having progressive disease (25% or more increase) were classified as non-responders. Median follow-up time for treatment of recurrent disease was 42.7 months.

Tissue processing

Primary tumor tissue processing was done as described previously (16). In summary, 20-60 cryostat sections of 30 μm, corresponding to 30-100 mg, were cut from frozen tissues for RNA isolation. To assess the amount of tumor cells relative to the amount of surrounding stromal cells, 5 μm sections were cut for hematoxylin and eosin staining, before, in between, and after cutting the sections for RNA isolation. For this study, only specimen with at least 30% tumor nuclei, distributed uniformly over at least 70% of the section area, were included.

RNA isolation, cDNA synthesis and quantification of mRNA

RNA isolation, cDNA synthesis, and quantification of mRNA and quality control checks were done as described in detail before (16). RNA samples without distinct rRNA peaks or failing to amplify efficiently with the housekeeper primer sets were excluded from this study. Real time quantitative RT-PCR was performed using an ABI Prism 7700 Sequence detection system (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) and Assay-on-Demand kits from Applied Biosystems. The following assays were used: Hs00182599_m1 (FGF17), Hs00241111_m1 (FGFR1), Hs00240796_m1 (FGFR2),

Hs00179829_m1 (FGFR3) and Hs00242558_m1 (FGFR4). We used a protocol as recommended by the manufacturer. Quantitative RT-PCR assays for EGFR and ERBB2 have previously been described (17). To enable comparison of the mRNA levels in different samples, values were normalized using the average expression levels of a set of housekeeping genes containing PBGD, HPRT and B2M. Levels of the target genes expressed relative to this housekeeping set were quantified as follows: mRNA target = $2^{(\text{mean Ct housekeeping} - \text{mean Ct target})}$ (16). Ct is defined as the detection threshold cycle number.

Statistical analysis

Computations were done with the use of STATA statistical package, release 9.2 (STATA Corp., College Station, TX). Differences in levels were assessed with the Mann-Whitney U test or Kruskal-Wallis test, including a Wilcoxon-type test for trend, when appropriate. In these tests, patient and tumor characteristics were used as grouping variables. The strengths of the associations between continuous variables were tested with the Spearman rank correlation (r_s). To reduce the skewness, most variables were log-transformed while ER-α and FGFR3 data were Box-Cox transformed. Logistic regression analysis was used to examine the relationship between the mRNA levels measured and clinical benefit of tamoxifen therapy. Odds ratios (ORs) were calculated and presented with their 95% confidence interval. The likelihood ratio test in logistic regression models was used to test for differences. The Cox proportional hazard model was used to calculate the hazard ratio (HR) and 95% confidence interval in the analyses of progression free survival (PFS) and post-relapse overall survival. The proportional hazards assumption was tested using Schoenfeld residuals. In most cases, tamoxifen treatment is started at the end of the disease free interval, except for those patients that were first treated with surgery and/or radiotherapy for local recurrence (n = 16). For all cases the starting point of PFS is defined as the start of the first line of systemic treatment with tamoxifen for recurrence. The endpoint is the first detection of progression of the disease. For visualization, levels of FGFR4 were divided into four equal parts. Survival curves were generated using the Kaplan & Meier's (18) method and the log-rank test was used to test for differences. A two-sided P-value of < 0.05 was considered statistically significant.

RESULTS

Correlations between biological factors

All tissue samples were analyzed for ER and PGR mRNA expression and none tested negative for ER-α mRNA in concordance with the selection of ER protein-positive samples (16). Spearman's rank correlation revealed associations ($P < 0.001$) between mRNA levels of FGFR4 and FGFR1 ($r_s = 0.23$), FGFR3 ($r_s = 0.29$), and PGR ($r_s = -0.31$). In addition, FGFR1

mRNA levels correlated with those of FGF17 ($r_s = 0.32$). ER-α mRNA levels correlated with those of PGR ($r_s = 0.22$), FGFR2 ($r_s = 0.34$), and FGFR3 ($r_s = 0.38$).

Association of biological factors with clinicopathological factors

In Table 3.1, the associations of the median mRNA levels of the factors measured with clinicopathological factors are shown. ER-α, FGFR2, FGFR3, and FGF17 mRNA levels were inversely related with tumor grade. Furthermore, ER-α mRNA expression levels were higher in tumors of postmenopausal patients and mRNA levels of FGF17 were inversely related with nodal status. PGR mRNA levels varied between histological subtypes, and mRNA levels of FGFR1 varied between the different categories of dominant site of relapse. None of the mRNA levels determined correlated with tumor size, disease-free interval (Table 3.1) or adjuvant systemic treatment (data not shown).

Uni- and multivariate analysis for clinical benefit

The main clinical endpoints of this study were the measurable effect of tamoxifen therapy on tumor size (clinical benefit) and the length of PFS of the patients after start of first-line therapy for recurrent disease. In univariate logistic regression analysis using log-transformed continuous variables, high expression levels of ER-α and PGR predicted a favorable clinical benefit (Table 3.2). In contrast, high levels of FGFR4 predicted a worse outcome for the patient (OR = 1.22, P = 0.009). For FGF17 and FGFR1-3 no significant associations with clinical benefit were observed (Table 3.2). The estimate for FGFR4 was similar when patients with a local recurrence were excluded. To further explore the predictive power of FGFR4, the patients were divided into four equal quarters, ranging from low to high FGFR4 mRNA levels. Compared with patients with low tumor mRNA levels of FGFR4 (OR set at 1.0), those with high FGFR4 mRNA levels showed a worse outcome (OR = 2.92, CI = 1.44-5.92, P = 0.003). Patients with intermediate levels of FGFR4 (Q2 and Q3) showed intermediate ORs (Table 3.2). The proportion of patients that experienced clinical benefit was 75% (54/72) for those with the lowest, and 51% (36/71) for those with the highest FGFR4 mRNA levels.

The predictive value of FGF17 and FGFR1-4 was further studied with multivariable logistic regression analysis (Table 3.2). For this analysis, we used the base multivariable model including traditional predictive factors (menopausal status, dominant site of relapse, disease-free interval, and ER), as described previously for a larger group of 691 patients treated with first-line tamoxifen for recurrent disease (19), with age and PGR added. The traditional prognostic factors 'nodal status', 'tumor size' and 'grade' were not included, because they did not have any predictive value as expected in an analysis for response to first line therapy for recurrent disease. 'Adjuvant chemotherapy' was also omitted, since the estimates of our base model were not different with adjuvant therapy in- or excluded. FGF17 and FGFR1-4 were separately added as transformed continuous

Table 3.1 Associations of biological factors with clinicopathological factors.

Clinicopathological factors	No. of patients[b]	Median (and 25% to 75% range) of biological factors after normalization to the housekeeper-set[a]													
		ER-α ($x10^1$)		PGR ($x10^0$)		FGFR1 ($x10^{-1}$)		FGFR2 ($x10^{-2}$)		FGFR3 ($x10^{-2}$)		FGFR4 ($x10^{-1}$)		FGF17 ($x10^{-4}$)	
Menopausal status[c]															
premenopausal	74	0.37	(0.18-0.72)	1.04	(0.33-2.06)	1.61	(0.80-3.47)	2.88	(1.21-6.77)	2.09	(0.57-5.65)	0.24	(0.12-0.61)	1.58	(0.61-3.31)
postmenopausal	211	0.88	(0.39-1.81)	0.62	(0.07-3.05)	1.94	(0.95-3.48)	3.95	(1.76-8.80)	2.57	(0.87-7.57)	0.35	(0.11-1.17)	1.36	(0.51-3.03)
		$P < 0.01$[d]		NS[d]		NS[d]		NS[d]		NS[d]		NS[d]		NS[d]	
Age[c] (years)															
≤ 40	18	0.50	(0.17-1.02)	0.99	(0.27-2.42)	1.40	(0.90-2.31)	4.61	(1.21-13.26)	2.92	(0.74-7.26)	0.26	(0.14-1.11)	1.29	(0.61-2.98)
41-55	93	0.40	(0.18-0.79)	1.01	(0.19-1.94)	2.27	(0.97-3.51)	3.14	(1.35-6.14)	2.04	(0.63-5.28)	0.25	(0.13-0.76)	1.69	(0.61-3.81
56-70	100	1.08	(0.38-1.88)	0.56	(0.06-3.31)	1.99	(1.00-3.93)	4.40	(1.57-6.69)	2.32	(0.77-6.69)	0.41	(0.11-1.31)	1.43	(0.51-2.81)
> 70	74	0.99	(0.66-1.97)	0.63	(0.07-3.05)	1.69	(0.75-3.00)	3.90	(2.11-10.76)	3.78	(1.21-10.18)	0.28	(0.10-0.77)	1.37	(0.39-2.99)
		$P < 0.01$[e]		NS[e]		NS[e]		NS[e]		$P = 0.03$[e]		NS[e]		NS[e]	
Tumor size															
≤ 2 cm	78	0.85	(0.31-1.49)	0.56	(0.08-2.06)	2.06	(0.77-3.51)	4.46	(1.71-10.19)	2.10	(0.82-7.39)	0.27	(0.14-0.62)	1.44	(0.61-3.06)
> 2- ≤5 cm	169	0.70	(0.31-1.53)	1.01	(0.13-3.27)	1.84	(1.00-3.48)	3.64	(1.71-7.11)	2.86	(0.76-7.32)	0.35	(0.12-0.88)	1.52	(0.67-3.14)
> 5 cm + pT4	38	0.54	(0.23-1.78)	0.42	(0.06-1.80)	1.29	(0.57-3.42)	2.51	(0.93-8.61)	1.95	(0.79-5.15)	0.36	(0.10-1.96)	61.61	(0.32-3.53)
		NS[e]		NS[e]		NS[e]		NS[e]		NS[e]		NS[e]		NS[e]	
Tumor grade															
good/moderate	35	1.06	(0.49-1.81)	0.79	(0.04-2.60)	2.09	(1.00-3.57)	5.16	(2.36-10.92)	4.88	(2.36-10.41)	0.61	(0.16-2.32)	2.43	(1.01-6.60)
poor	158	0.58	(0.25-1.14)	0.66	(0.08-2.55)	1.67	(0.77-3.32)	3.42	(1.33-7.11)	2.02	(0.63-5.34)	0.39	(0.12-1.08)	1.28	(0.46-2.25)
		$P = 0.01$[d]		NS[d]		NS[d]		$P = 0.04$[d]		$P < 0.01$[d]		NS[d]		$P = 0.01$[d]	
Histological type															
IDC	163	0.78	(0.30-1.53)	0.47	(0.06-2.23)	1.84	(0.90-3.80)	4.13	(1.71-8.85)	2.78	(0.89-7.39)	0.35	(0.13-1.27)	1.46	(0.53-3.16)
ILC	28	0.72	(0.31-1.71)	1.46	(0.42-4.36)	2.10	(1.06-3.39)	3.80	(1.92-8.64)	2.37	(0.67-7.31)	0.16	(0.09-0.34)	2.01	(0.48-4.23)
DCIS + IDC	24	0.51	(0.17-0.97)	0.98	(0.33-1.22)	2.46	(0.97-3.61)	2.84	(1.12-8.24)	1.93	(0.69-6.57)	0.47	(0.16-0.64)	1.22	(0.45-2.36)
		NS[e]		$P = 0.04$[e]		NS[e]		NS[e]		NS[e]		NS[e]		NS[e]	

Clinicopathological factors	No. of patients[b]	Median (and 25% to 75% range) of biological factors after normalization to the housekeeper-set[a]													
		ER-α ($x10^{1}$)		PGR ($x10^{0}$)		FGFR1 ($x10^{-1}$)		FGFR2 ($x10^{-2}$)		FGFR3 ($x10^{-2}$)		FGFR4 ($x10^{-1}$)		FGF17 ($x10^{-4}$)	
Nodal status															
N0	120	0.86	(0.33-1.63)	0.40	(0.07-2.21)	2.16	(1.00-4.02)	4.41	(1.73-9.00)	2.78	(0.90-8.80)	0.32	(0.13-0.68)	1.85	(0.79-4.39)
N1-3	66	0.61	(0.26-1.43)	1.05	(0.13-3.10)	1.56	(0.96-2.60)	3.41	(1.52-7.11)	2.45	(0.60-4.91)	0.45	(0.16-1.17)	1.27	(0.54-2.27)
N > 3	82	0.57	(0.27-1.26)	1.04	(0.23-2.64)	1.68	(0.75-3.03)	3.40	(1.30-8.65)	2.08	(0.75-6.81)	0.24	(0.08-1.40)	0.92	(0.32-2.68)
		NS[e]		NS[e]		NS[e]		NS[e]		NS[e]		NS[e]		P < 0.01[e]	
DSR															
LRR	32	0.47	(0.20-1.48)	0.47	(0.11-2.73)	1.18	(0.53-2.06)	2.36	(1.27-4.93)	2.06	(0.57-8.64)	0.51	(0.23-2.03)	1.29	(0.46-4.47)
bone	146	0.70	(0.30-1.29)	0.75	(0.09-2.52)	1.93	(0.99-3.30)	3.79	(1.75-8.47)	2.54	(0.83-7.04)	0.26	(0.12-1.01)	1.32	(0.59-2.74)
viscera	107	0.77	(0.34-1.79)	0.95	(0.07-3.28)	2.16	(0.81-4.54)	4.37	(1.41-8.85)	2.46	(0.70-7.16)	0.35	(0.11-075)	1.74	(0.51-3.35)
		NSe		NS[e]		P = 0.01[e]		NS[e]		NS[e]		P = 0.05[e]		NS[e]	
DFI															
≤ 1 yr	74	0.72	(0.36-1.19)	0.49	(0.07-1.67)	2.17	(0.81-3.30)	2.62	(1.02-7.81)	2.17	(0.82-6.14)	0.36	(0.15-1.54)	1.14	(0.43-2.23)
> 1-3 yr	125	0.73	(0.30-1.43)	0.85	(0.08-2.41)	1.79	(1.00-3.42)	4.39	(1.71-8.80)	2.62	(0.95-7.26)	0.35	(0.12-0.75)	1.38	(0.61-3.33)
> 3 yr	86	0.63	(0.32-1.79)	1.07	(0.17-3.63)	1.63	(0.81-3.57)	3.79	(1.82-7.88)	2.32	(0.60-8.46)	0.24	(0.08-0.89)	1.66	(0.74-3.76)
		NS[e]		NS[e]		NS[e]		NS[e]		NS[e]		NS[e]		NS[e]	

[a] Due to different assay conditions and amplicon lengths, absolute values of the biological factors can only be compared within a gene-assay.

[b] Because of others and unknowns, numbers do not always add up to 285.

[c] At start of first-line therapy for recurrent disease.

[d] P for Mann-Whitney U test,.

[e] P for Kruskal-Wallis test, including a Wilcoxon-type test for trend when appropriate.

Abbreviations: ER-α, estrogen receptor alpha; PGR, progesterone receptor, FGFR fibroblast growth factor receptor; IDC, infiltrating ductal carcinoma; ILC, infiltrating lobular carcinoma; DCIS, ductal carcinoma in situ; DSR, dominant site of relapse; LRR, local regional relapse; DFI, disease-free interval; NS, not significant.

Table 3.2 Uni- and multivariate logistic regression analysis for clinical benefit of patients receiving first-line tamoxifen therapy.

			Univariate analysis[a]			Multivariate analysis[a,b]		
Factor	No of patients	Clinical benefit	P	OR	95% CI	P	OR	95% CI
	285	63%				**Base model**		
Menopausal status[c]			0.038			0.47		
premenopausal	74	53%		1			1	
postmenopausal	211	66%		0.57	0.33-0.97		0.73	0.30-1.73
Age[c] years			0.16			0.86		
≤ 40	18	61%		1			1	
41-55	93	54%		1.35	0.48-3.79		1.48	0.48-4.55
56-70	100	67%		0.77	0.27-2.18		1.23	0.32-4.76
>70	74	69%		0.71	0.0.24-2.06		1.13	0.28-4.55
DSR			0.20			0.18		
LRR	32	75%		1			1	
bone	146	59%		2.09	0.88-4.97		2.36	0.91-6.10
viscera	107	64%		1.65	0.68-4.03		2.25	0.84-6.04
DFI			< 0.001			< 0.001		
≤ 1 yr	74	42%		1			1	
1-3 yrs	125	70%		0.31	0.17-0.57		0.30	0.16-0.56
> 3 yrs	86	71%		0.30	0.15-0.57		0.30	0.15-0.60
ER-α	285		< 0.001	0.81	0.72-0.91	0.004	0.83	0.73-0.94
PGR	285		0.044	0.86	0.74-1.00	0.317	0.92	0.78-1.08
						Additions to the base model[d]		
FGF17	285		0.905	0.99	0.83-1.18	0.703	1.04	0.86-1.26
FGFR1	285		0.761	1.03	0.84-1.28	0.787	1.03	0.81-1.31
FGFR2	285		0.732	0.96	0.77-1.20	0.178	1.20	0.92-1.56
FGFR3	285		0.927	1.01	0.82-1.25	0.055	1.28	0.99-1.65
FGFR4	285		0.009	1.22	1.05-1.41	0.031	1.21	1.02-1.43
FGFR4								
Q1	72	75%		1			1	
Q2	71	58%	0.030	2.20	1.08-4.47	0.089	1.96	0.90-4.27
Q3	71	68%	0.329	1.44	0.69-2.98	0.535	1.28	0.58-2.81
Q4	71	51%	0.003	2.92	1.44-5.92	0.021	2.61	1.15-5.87

[a] The probability of non-response has been modeled.

[b] Biological factors were separately introduced as transformed continuous variable to the base multivariable model that included the factors menopausal status, age, DSR dominant site of relapse, DFI disease-free interval, and ER-α estrogen receptor alpha and PGR progesterone receptor mRNA levels as transformed continuous variables.

[c] At start of first-line therapy for recurrent disease.

[d] Factors added separately to the base model.

Abbreviations: OR, odds ratio; CI, confidence interval

Table 3.3 Uni- and multivariate Cox regression analysis for progression-free survival of patients receiving first-line tamoxifen therapy.

		Univariate analysis			Multivariate analysis[a]		
Factor	No of patients	P	HR	95% CI	P	HR	95% CI
	285				**Base model**		
Menopausal status[b]		0.14			0.081		
premenopausal	74		1			1	
postmenopausal	211		0.81	0.62-1.07		1.46	0.95-2.23
Age[b] years		0.025			0.051		
≤ 40	18		1			1	
41-55	93		0.76	0.46-1.26		0.60	0.35-1.03
56-70	100		0.61	0.36-1.01		0.42	0.22-0.80
> 70	74		0.51	0.30-0.86		0.38	0.20-0.74
DSR		0.20			0.35		
LRR	32		1			1	
bone	146		1.43	0.95-2.17		1.26	0.81-1.94
viscera	107		1.30	0.85-1.99		1.38	0.88-2.18
DFI		0.005			0.022		
≤ 1 yr	74		1			1	
1-3 yr	125		0.72	0.54-0.97		0.74	0.54-1.00
> 3 yr	86		0.58	0.42-0.80		0.62	0.44-0.87
ER-α	285	< 0.001	0.91	0.86-0.95	0.028	0.94	0.88-0.99
PGR	285	0.002	0.89	0.83-0.96	0.024	0.91	0.85-0.99
					Additions to the base model[c]		
FGF17	285	0.802	1.01	0.93-1.10	0.474	1.03	0.95-1.13
FGFR1	285	0.077	1.10	0.99-1.23	0.054	1.12	1.00-1.26
FGFR2	285	0.090	0.90	0.79-1.02	0.394	0.94	0.83-1.08
FGFR3	285	0.233	0.94	0.84-1.04	0.656	1.03	0.91-1.16
FGFR4	285	< 0.001	1.18	1.09-1.27	< 0.001	1.17	1.08-1.27
FGFR4							
Q1	72		1			1	
Q2	71	0.208	1.25	0.88-1.77	0.468	1.15	0.79-1.67
Q3	71	0.357	1.18	0.83-1.66	0.470	1.14	0.80-1.62
Q4	71	<.001	2.24	1.58-3.16	<.001	2.11	1.43-3.12

[a] Biological factors were separately introduced as log-transformed continuous variable to the base multivariable model that included the factors menopausal status, age, DSR dominant site of relapse, DFI disease-free interval, and ER-α estrogen receptor alpha and PGR progesterone receptor mRNA levels as transformed continuous variables.

[b] At start of first-line therapy for recurrent disease.

[c] Factors added separately to the base model.

Abbreviations: HR, hazard ratio

variables to the base model. These analyses showed that the predictive value of FGFR4 (OR = 1.21, P = 0.031) for clinical benefit was independent from the traditional predictive factors of the base model (Table 3.2).

Analysis for progression-free and post-relapse overall survival

In univariate analysis using log-transformed continuous variables, high tumor levels of ER-α and PGR mRNA were correlated with longer PFS and thus with better outcome for the patient (Table 3.3). On the other hand, high mRNA levels of FGFR4 were significantly associated with shorter PFS (log-transformed continuous, HR = 1.18, P < 0.001). To allow visualization by Kaplan-Meier plots, patients were divided into four equal quarts according to their FGFR4 mRNA levels. Patients having low FGFR4 mRNA levels (quarter 1) had a median PFS of more than twice the PFS of patients having high FGFR4 mRNA levels (quarter 4) (14 vs. 6 months Figure 3.1A). Post-relapse overall survival (Figure 3.1B) was also significantly better in patients with low FGFR4 mRNA levels. In multivariable analysis, the predictive value of FGFR4 was independent of the traditional predictive factors (Table 3.3). When divided in quarts, the hazards ratios compared with the lowest quartile (Q1) were 1.15 (Q2), 1.14 (Q3) and 2.11 (Q4). The proportional hazards assumption was not violated for FGFR4.

DISCUSSION

Recently, we have identified FGF17 in a functional screen for genes responsible for tamoxifen resistance in a human breast cancer cell line (5). FGF17 is predominantly expressed in the brain and the nervous system and no data were available for its expression in breast cancer until now. In contrast, other FGFs and the FGFRs have been reported to be expressed and to have a potential role in breast cancer (reviewed by 20, 21). Furthermore, FGF2 has been linked to tumor aggressiveness (22, 23) and FGFR3 and FGF5 to metastatic preferences of breast cancer (24, 25).

In the current exploratory study, quantitative RT-PCR was used to evaluate whether mRNA expression levels of FGF17 and the receptors FGFR1-4 in primary tumors can predict the outcome of first-line tamoxifen treatment. The endpoints of this study were clinical benefit of the therapy and PFS in patients with recurrent breast cancer. Because of the retrospective nature of this study, we have defined the type of response strictly beforehand. The size of the metastases or the occurrence of new lesions was used as objective measures of treatment effect. We included only hormone-naive patients to exclude acquired therapy resistance to tamoxifen.

Our study shows that FGF17 mRNA levels did not associate with clinical benefit and PFS of patients treated with tamoxifen. In contrast, increasing levels of its receptor FGFR4

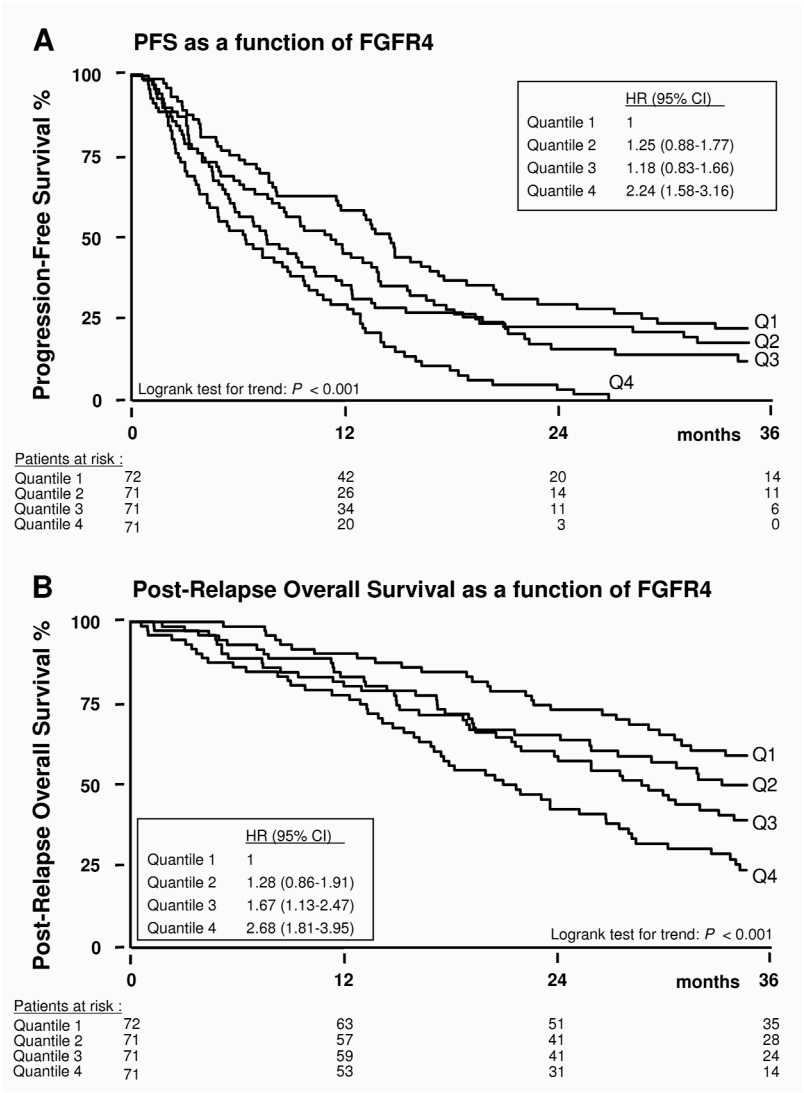

Figure 3.1 Kaplan-Meier curves of progression-free survival (panel A) and post-relapse overall survival (panel B) for advanced breast cancer patients after start of first-line tamoxifen therapy as a function of the FGFR4 mRNA levels in quarters. Patients at risk are indicated.

were related with a higher probability of tamoxifen failure. In multivariable analysis, high tumor levels of FGFR4 provided additional predictive information, independent of the traditional predictive factors menopausal status, dominant site of relapse, disease free interval, ER, and PGR status. Moreover, addition of the mRNA levels of EGFR and ERBB2 (HER2) as variables to the base model did not alter the estimates for FGFR4 for PFS or clinical benefit (Table 3.4). No significant correlation was observed with disease-free survival in this patient series (Table 3.1), nor in an independent set of 604 node-negative

Table 3.4 FGFR4 is independent of EGFR and ERBB2.

	Clinical Benefit[a]			Progression-Free Survival[b]		
Factor	P	OR	95% CI	P	HR	95% CI
EGFR[c]	0.186	0.84	0.64-1.09	0.468	0.96	0.85-1.08
ERBB2[d]	0.545	1.28	0.57-2.86	0.003	1.81	1.22-2.69
FGFR4[c]	0.045	1.20	1.00-1.43	0.002	1.15	1.05-1.25

[a] Multivariable logistic regression analysis for clinical benefit of patients receiving first-line tamoxifen therapy with simultaneous addition of the factors and corrected for the base model as detailed in Table 2.

[b] Multivariable Cox regression analysis for progression-free survival of patients receiving first-line tamoxifen therapy with simultaneous addition of the factors and corrected for the base model as detailed in Table 3.

[c] Factor added as log-transformed continuous variable.

[d] Factor added as dichotomized variable representing high (14% of the patients) and low levels of mRNA.

breast cancer patients (data not shown), indicating that FGFR4 mRNA levels are not merely associated with prognosis. For FGFR4, gene amplification and relatively high expression levels have been found in breast cancer (26, 27). In addition, a germ line polymorphism in this gene, resulting in expression of FGFR4 variants containing either glycine (Gly^{388}) or arginine (Arg^{388}) at codon 388, has been identified (28). However, there has been some debate whether the Arg^{388} allele is relevant for breast cancer prognosis (28-32). Furthermore, Thussbas *et al.* (32) reported a survival difference favoring FGFR4 wild type in patients treated with adjuvant chemotherapy. In addition, no significant survival difference between the variants was seen in patients treated with adjuvant endocrine therapy. Future analysis of this polymorphism in our patient series may establish its predictive value.

FGFR4 adds to an expanding list of biological factors which provide predictive information for tamoxifen treatment of breast cancer (33). Prominent examples are uPA/PAI-1 (19, 34), cyclin E (35), HOXB13/IL17BR (36, 37), the 81 gene signature (38), and BCAR1, a gene identified in another functional screen (39).

The question whether FGFR4 association with development of tamoxifen resistance is caused in a direct manner remains to be solved. The observation that FGF17 in epithelial lineages mainly activates FGFR4 (40), supports the theory that the biological effects seen in our functional screen were caused by activation of this particular receptor. In concordance with this hypothesis, we have confirmed expression of FGFR4 (at levels comparable with the housekeepers) and the other FGFR family members in our cell model using quantitative RT-PCR analysis (unpublished results). The absence of association between FGF17 and FGFR4 levels in the breast tumors may be explained by the balance between the receptor concentration and the amount and affinity of all ligands. Activation of FGFRs may lead to activation of the MAPK and the PI3K/AKT pathways (41). Increasing evidence indicates that changes occurring in growth factor signaling pathways, as currently well documented for EGFR and ERBB2, may dramatically influence

steroid hormone action and may be critical to anti-hormone-resistant breast cancer cell growth (42-48).

In conclusion, the established association between FGFR4 mRNA levels and clinical benefit of therapy suggests that FGFR4 plays a role in the biological response of the tumor to tamoxifen treatment. As altered FGF signaling causes tamoxifen resistance *in vitro*, this pathway could be a valuable target in the treatment of breast cancer patients resistant to endocrine treatment.

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CHAPTER 4

TSC22D1 AND PSAP PREDICT CLINICAL OUTCOME OF TAMOXIFEN TREATMENT IN PATIENTS WITH RECURRENT BREAST CANCER

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ABSTRACT

Purpose: Two genes, TSC22 domain family, member 1 (TSC22D1) and prosaposin (PSAP) were identified in an *in vitro* functional screen for genes having a causative role in tamoxifen resistance. These genes were also present in our previously established 81-gene signature for resistance to first-line tamoxifen therapy. The aim of this study was to investigate the predictive value of these genes for tamoxifen therapy failure in patients with recurrent breast cancer. *Experimental Design:* The mRNA levels of TSC22D1 and PSAP were analyzed by quantitative real-time polymerase chain reaction (qRT-PCR) in 223 estrogen receptor-positive primary breast tumors of patients with recurrent disease treated with first-line tamoxifen therapy. The main objective of this study was the length of progression-free survival (PFS). *Results:* High mRNA levels of TSC22D1 and PSAP were significantly associated with shorter PFS and both were independent of the traditional predictive factors (HR = 1.30, 95% CI = 1.04-1.64 P = 0.023; and HR = 1.40, 95% CI = 1.03-1.88, P = 0.029, respectively). In multivariate analysis, patients with high mRNA levels of both genes associated significantly with no clinical benefit (OR = 0.19, 95% CI = 0.06-0.62, P = 0.006) and had the shortest PFS (HR = 2.05, 95% CI = 1.29-3.25, P = 0.002). *Conclusion:* These results confirm our previous *in vitro* and tumor-related findings and are indicative for the failure of tamoxifen treatment in breast-cancer patients. Both TSC22D1 and PSAP are associated with clinical outcome and may have a functional role in therapy resistance.

INTRODUCTION

Breast cancer is the most common malignancy among women in the Western world. For more than 30 years, the anti-estrogen tamoxifen has been used for the treatment of estrogen receptor (ER)-positive breast cancer. However, only half of the patients who have ER-positive tumors respond to tamoxifen therapy, while the other half show intrinsic resistance. Moreover, patients who initially respond to therapy, will ultimately acquire resistance during long-term treatment.

In the last decades, various studies have been performed to identify biological factors that predict the success of tamoxifen treatment (1). Several molecular signatures have been reported to associate with tamoxifen therapy efficacy. A 21-gene (2) and a 2-gene signature, HOXB13/IL17BR (3), were correlated with disease-free survival in patients treated with tamoxifen in the adjuvant setting. In a genome-wide microarray study, we defined an 81-gene signature for tamoxifen therapy failure in patients with advanced disease (4). Although these gene signatures classify for prediction of clinical outcome, they do not necessarily identify those genes that are functionally responsible for treatment resistance.

Therefore, we performed a different approach, namely retroviral transduction of cDNA libraries, to identify genes causing tamoxifen resistance. In this *in vitro* functional screen, a set of 73 different genes has been identified. Using stringent selection criteria 7 genes have been reported to cause tamoxifen resistance (5). The latter included epidermal growth factor receptor (EGFR), Neuregulin 1 (NRG1), platelet-derived growth factor receptor-α (PDGFRA), platelet-derived growth factor receptor-β (PDGFRB), colony-stimulating factor 1 receptor (CSF1R), fibroblast growth factor 17 (FGF17), and breast cancer anti-estrogen resistance 4 (BCAR4). From these, EGFR, BCAR4, and NRG1 have already been related with tamoxifen resistance. High levels of EGFR and BCAR4 promote anti-estrogen resistant proliferation of breast-cancer cells *in vitro* (5-7). High expression levels of NRG1 changed MCF-7 breast cancer cells into an estrogen-independent and antiestrogen-resistant state, which was later mimicked in a transgenic mice model (8). In addition, HER2 an established marker and a proven target for patients resistant to tamoxifen therapy, was amongst the remaining 66 discovered genes. However, the remaining genes need further validation to establish their role in resistance *in vitro* and their association with therapy failure in clinical breast cancer.

We propose that those genes identified with two independent approaches are interesting candidates as predictive markers and/or targets for treatment. Interestingly, only two genes, TSC22D1 (OMIM 607715; 13q14) and PSAP (OMIM 176801; 10q21-q22), were identified that were both highly expressed in tumors resistant to tamoxifen in our microarray study and were recovered from our functional screen.

In the current study, we investigated the clinical relevance of the TSC22D1 and PSAP in tamoxifen resistance with a third platform. To this end, gene mRNA levels were measured by qRT-PCR in 223 ER-positive primary breast tumors from patients who developed recurrent disease that was treated with tamoxifen as first-line therapy.

PATIENTS AND METHODS

Patients

This retrospective study has been approved by the medical ethics committee of the Erasmus MC Rotterdam, The Netherlands (MEC 02.953). Total RNA was isolated from frozen primary breast tumors of women who entered the clinic between 1981 and 1995 and from whom detailed clinical follow-up information was available (9, 10). The present study, in which coded tumor tissues were used, was performed in accordance with the Code of Conduct of the Federation of Medical Scientific Societies in the Netherlands (http://www.fmwv.nl). Specimens were excluded when patients had received (neo)adjuvant hormonal therapy, when there was insufficient frozen tumor material, less than 30% epithelial tumor cells, poor RNA quality, or missing values for TSC22D1 or PSAP mRNA expression levels. In total, ER-positive breast tumors of 223 patients who developed advanced disease treated with first-line tamoxifen therapy were included in this study. ER and progesterone receptor (PgR) protein status of the tumors was determined by routine ligand-binding assays or enzyme immunoassays. Eighty-five patients (38%) underwent breast conserving lumpectomy and 138 patients (62%) modified mastectomy. Ninety-two percent of the patients underwent an axillary-node dissection (n = 206) and 110 patients were node positive (49%) at the time of surgical removal of the primary tumor.

All patients, including 13 patients with a local recurrence, were treated with first-line tamoxifen therapy (40 mg daily) for advanced disease. Adjuvant radiotherapy was given to 58% of the patients and 34 patients (15%) were treated with adjuvant chemotherapy (16 patients anthracyclin-based (FAC/FEC) and 18 patients non-anthracyclin-based (CMF)). Median time to recurrence was 26 months. Median follow-up for recurrent disease of patients alive and treated with tamoxifen was 44 months. The median age of the patients at the time of primary surgery was 59 years (range, 26-89 years) and at the start of first-line tamoxifen therapy for recurrent disease 62 years (range, 29-90 years). In total, 168 patients were postmenopausal at start of therapy. Twenty-five patients had metastasis at diagnosis or developed distant metastasis (including supraclavicular lymph node metastasis) within 1 month after primary surgery.

The type of response to tamoxifen therapy was recorded as defined by standard Union Internationale Contre Cancer criteria (11). No clinical benefit occurred in 91 patients

(41%) of whom 81 patients had progressive disease and 10 patients showed stable disease shorter than 6 months. One hundred thirty-two patients (59%) showed clinical benefit from first-line tamoxifen therapy, of whom 11 patients showed a complete remission, 34 patients showed partial remission, and 87 patients showed stable disease longer than 6 months.

RNA isolation and quantitative RT-PCR

Tissue processing, RNA isolation, cDNA synthesis and quantitative real-time reverse-transcriptase polymerase chain reaction were performed as described previously (9). The qRT-PCRs were performed on an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands), according to the recommended protocol. Commercially available Assay-on-Demand kits (Applied Biosystems) were used for TSC22D1 (Hs00394659_m1) and PSAP (Hs00358165_m1). Forty rounds of amplification were performed according to the supplier's protocol and at the end of the amplification fluorescent signals of the Taqman probes were used to generate Cycle threshold (Ct) values from which mRNA expression levels were calculated. Expression levels of TSC22D1 and PSAP were normalized against average expression levels of three housekeeping genes, i.e., porphobilinogen deaminase (PBGD), hypoxanthine-guanine phospho-ribosyltransferase (HPRT) and β-2-microglobulin (B2M) (9).

Data analysis and statistics

Differences in mRNA levels were assessed with the Mann-Whitney U test or Kruskal-Wallis test when appropriate. In these tests, patient and tumor characteristics were used as grouping variables. Associations between continuous variables were tested with the Spearman rank correlation (R_s). Cox proportional-hazards models were applied to compute the hazard ratio (HR), which correlates expression levels of the variables with progression-free survival. Progression-free survival was defined as the time between start of treatment with tamoxifen as first-line systemic therapy for recurrent disease and the occurrence of disease progression. In multivariate analysis, Cox regression analysis was applied to determine whether TSC22D1 and PSAP had a predictive value and was independent when added to the base model of the traditional predictive factors age, menopausal status, disease-free interval (DFI), dominant site of relapse (DSR), and log ER and log PgR mRNA levels (12). The proportional hazards assumption was not violated for TSC22D1 and PSAP in any of these analyses. Logistic-regression analysis was performed to calculate the odds ratio (OR) that defines the relation between expression levels and clinical benefit of tamoxifen therapy. Both HR and OR were calculated on log-transformed variables and were represented with their 95% confidence interval (95% CI).

Only when the test for trend of a continuous variable was statistically significant a search for a cutoff point was considered justified. To define cutoff points, we used iso-

tonic regression analysis to find the points where the monotonic relationship between the measured level and the hazard rate showed a distinct change. On the basis of these cutoff points, survival curves were generated using the method of Kaplan and Meier and a log-rank test was used to test for differences. Computations were performed with the STATA statistical package, release 10 (STATA Corp., College Station, TX). All P-values were two-sided and $P < 0.05$ was considered statistically significant.

RESULTS

Associations of TSC22 and PSAP with clinicopathological factors

In this study, TSC22D1 and PSAP mRNA expression levels were measured in 223 primary breast tumors using qRT-PCR. Median expression levels of TSC22D1 and PSAP, their interquartile range, and their association with patient and tumor characteristics are shown in Table 4.1 TSC22D1 and PSAP expression levels did not have a relationship with menopausal status, tumor size, nodal status, tumor grade, nor with histological subtype (Table 4.1). All tissues were ER positive at the protein level and in agreement none of the samples tested negative for ER-α mRNA levels (9). Both TSC22D1 and PSAP mRNA levels had an inverse correlation with ER and PgR at mRNA levels (Rs = -0.06 and -0.16 for TSC22D1 and Rs = -0.03 and -0.09 for PSAP, respectively). At protein levels, these inverse correlations were significant (Rs = -0.16 and -0.26 for TSC22D1 and Rs = -0.15 and -0.17 for PSAP, respectively). Moreover, at mRNA levels TSC22D1 was significantly ($P < 0.001$) correlated with PSAP ($R_s = 0.33$), EGFR ($R_s = 0.37$), and ERBB2 ($R_s = 0.24$). Finally, PSAP had a significant ($P < 0.001$) correlation with EGFR ($R_s = 0.22$), but not with ERBB2.

Association of TSC22D1 and PSAP expression levels with progression-free survival and clinical benefit

Clinical endpoint of this study was progression after start of first-line tamoxifen therapy. TSC22D1 and PSAP mRNA levels, as univariate continuous log-transformed variables, were significantly associated with shorter PFS (HR = 1.39, 95% CI = 1.12-1.72, P = 0.003 and HR = 1.51, 95% CI = 1.13-2.02, P = 0.006, respectively) (Table 4.2). TSC22D1 expression was also significantly associated with clinical benefit to tamoxifen therapy (OR = 0.64, 95% CI = 0.42-0.98, P = 0.042), while PSAP was not.

The significant findings of TSC22D1 and PSAP for PFS as continuous variable in univariate analysis justified the search for predictive cutoff points. Cutoff points for TSC22D1 and PSAP were 0.042 and 0.378, respectively. Patient groups with the highest levels of TSC22D1 or PSAP had a worse outcome for first-line tamoxifen treatment (HR = 1.72, 95% CI = 1.26-2.33, $P < 0.001$; HR= 1.79, 95% CI = 1.23-2.59, P = 0.002, respectively) (Table 4.2). The predictive values of dichotomized TSC22D1 and PSAP are visualized

Table 4.1 Associations of TSC22D1 and PSAP mRNA levels with clinicopathological factors

			TSC22D1			*PSAP*		
	No. patients	**%**	**Median**	**Δ[§]**	**P ***	**Median**	**Δ[§]**	**P ***
Age (years)[†]					0.033 ‖			0.215 ‖
≤40	23	10	0.074	0.048		0.624	0.357	
41-55	74	33	0.061	0.056		0.668	0.611	
56-70	82	37	0.063	0.068		0.711	0.607	
>70	44	20	0.046	0.059		0.784	0.581	
Menopausal status[†]					0.319 ††			0.670 ††
Premenopausal	73	33	0.069	0.047		0.648	0.592	
Postmenopausal	150	67	0.060	0.068		0.741	0.565	
Tumor size					0.154 ‡			0.236 ‡
pT1, ≤2cm	63	28	0.061	0.058		0.744	0.557	
pT2, >2-5cm	130	58	0.066	0.061		0.692	0.592	
pT3, >5cm + pT4	30	13	0.049	0.059		0.669	0.669	
Lymph nodes involved					0.652 ‡			0.111 ‡
0	98	47	0.062	0.068		0.741	0.594	
1-3	52	25	0.061	0.056		0.601	0.513	
>3	58	28	0.059	0.06		0.693	0.557	
Grade					0.912 ‡			0.763 ‡
Poor	125	56	0.061	0.065		0.704	0.664	
Unkown	71	32	0.063	0.056		0.728	0.568	
Good/moderate	27	12	0.059	0.064		0.652	0.338	
Histological type					0.411 ‡			0.187 ‡
IDC	134	60	0.061	0.065		0.733	0.572	
ILC	24	11	0.043	0.041		0.654	0.371	
DCIS + IDC	13	6	0.068	0.016		0,637	0,580	
other/unknown	52	23	0.068	0.063				
PgR protein status[**]					<0.001 ‖			0.010 ‖
PgR low	37	17	0.089	0.094		0.857	0.695	
PgR high	183	83	0.059	0.052		0.688	0.581	
Response Tamoxifen first-line treatment					0.021 ‡			0.268 ‡
Complete response	11	5	0.037	0.034		0.473	0.830	
Partial response	34	15	0.045	0.073		0.744	0.603	
Stable disease > 6 months	87	39	0.060	0.051		0.649	0.521	
Stable disease ≤ 6 months	10	5	0.098	0.045		0.919	0.673	
Progressive disease	81	36	0.063	0.063		0.758	0.532	

[§] Interquartile range (q75-q25).

[*] Two-sided P-value.

[†] at primary surgery

‖ Spearman rank correlation.

[††] Mann-Whitney U test.

[‡] Kruskal-Wallis test

[**] In 3 samples PgR status was unknown

Table 4.2 Cox uni- and multivariate analysis for progression-free survival of patients receiving first-line tamoxifen therapy

			Univariate analysis				Multivariate analysis			
Factor of base model	**No. of patients**	**%**	**HR**	**95% CI**		**P**	**HR**	**95% CI**		**P**
Age (years) *						0.041				0.041
≤40	14	6	1				1			
41-55	69	31	0.74	0.41	1.32		0.63	0.34	1.16	
56-70	81	36	0.65	0.37	1.16		0.48	0.23	1.00	
>70	59	26	0.48	0.26	0.87		0.35	0.16	0.75	
Menopausal status *						0.337				0.112
Premenopausal	55	25	1				1			
Postmenopausal	168	75	0.85	0.62	1.17		1.47	0.91	2.36	
Disease-Free-Interval						<0.001				<0.001
≤ 1year	60	27	1				1			
1-3 years	99	44	0.63	0.45	0.87		0.58	0.42	0.82	
>3 years	64	29	0.46	0.31	0.67		0.44	0.30	0.65	
Dominant site of relapse						0.446				0.550
viscera	84	38	1				1			
Bone	119	53	1.05	0.78	1.41		0.91	0.66	1.24	
LRR	20	9	0.76	0.45	1.29		0.74	0.42	1.30	
ER-α mRNA level	223	100	0.89	0.83	0.95	<0.001	0.91	0.84	0.98	0.014
PgR mRNA level	223	100	0.89	0.83	0.96	0.002	0.89	0.82	0.97	0.005
Factors analysed							**Additions to the base model**			
TSC22D1										
Continuous variable	223	100	1.39	1.12	1.72	0.003	1.30	1.04	1.64	0.023
Cutoff point for PFS ‡										
low	70	31	1				1			
high	153	69	1.72	1.26	2.33	<0.001	1.60	1.16	2.19	0.004
PSAP										
Continuous variable	223	100	1.51	1.13	2.02	0.006	1.40	1.03	1.88	0.029
Cutoff point for PFS ‡										
low	41	18	1				1			
high	182	82	1.79	1.23	2.59	0.002	1.63	1.12	2.37	0.001
TSC22D1 & PSAP										
Cutoff point for PFS ‡										
Group 1 (both genes low)	27	12	1				1			
Group 2 (one gene low)	57	26	1.54	0.93	2.54	0.09	1.49	0.9	2.47	0.12
Group 3 (both genes high)	139	62	2.29	1.46	3.61	<0.001	2.05	1.29	3.25	0.002

* At start of first-line therapy for recurrent disease.

‡ cutoff points were determined at 0.042 for *TSC22D1* and 0.378 for *PSAP*

with Kaplan-Meier curves (Figure 4.1). The differences in median PFS were 7.5 and 7 months between patients with high and patients with low expression levels of TSC22D1 or PSAP, respectively. Furthermore, TSC22D1 dichotomized at the cutoff point was also significantly associated with clinical benefit to tamoxifen therapy (OR = 0.38, 95% CI = 0.20-0.71, P = 0.002).

In multivariate analysis, both factors analyzed as continuous and dichotomized variable were significantly associated with PFS (Table 4.2), showing that they are independent from the traditional predictive factors (age, menopausal status, DSR, DFI, ER and PgR levels). When adjuvant chemotherapy was added to the base model, the estimates for TSC22D1 and PSAP did not change indicating that possible chemical castration prior to endocrine therapy has no significant impact on these results. In multivariate analysis for clinical benefit, dichotomized TSC22D1 levels showed a significant relation with clinical benefit (OR = 0.39, 95% CI = 0.19-0.78, P = 0.008).

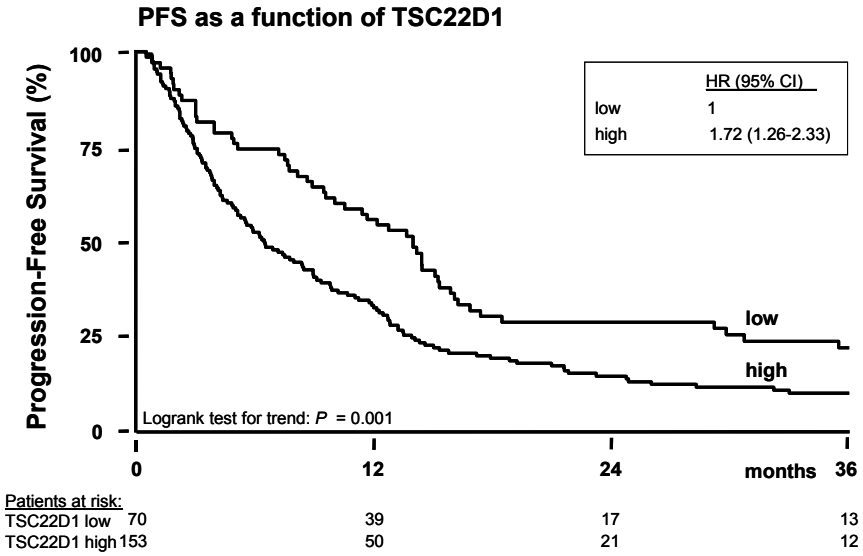
In a subsequent exploratory analysis, we divided our patient group into three subsets on the basis of their mRNA levels for both TSC22D1 and PSAP (both genes low (group 1), one gene low (group 2), and both genes high (group 3)). Compared with the patients having low mRNA levels for both genes (HR set at 1.0), those with high mRNA levels for both genes (62% of the patients) had a significantly shorter PFS (HR = 2.29, 95% CI = 1.46-3.61, P < 0.001). Patients having low levels for one gene (26% of the patients) showed an intermediate HR (1.54, P = 0.09, 95% CI = 0.93-2.54) (Table 4.2). The difference in median PFS between patient groups having high versus low levels for both genes was 9 months (Figure 4.2). Multivariate analysis for PFS (Table 4.2) and clinical benefit (OR intermediate = 0.30, 95% CI = 0.08-1.05, P = 0.06; OR high = 0.19, 95% CI = 0.06-0.62, P = 0.006) showed that these results are independent from traditional predictive factors.

DISCUSSION

Recently, we performed a genome wide screen in breast tumor samples to uncover a set of markers predictive for the type of response to endocrine therapy (4) and a functional screen using retroviral transduction of breast cancer cells for finding genes involved in tamoxifen resistance (5). In the first approach, we have identified 81 genes predicting resistance to tamoxifen therapy and in the latter approach we found 73 candidate genes possibly having a causative role in tamoxifen resistance. Combining the results of both approaches, two overlapping genes, PSAP and TSC22D1, were identified.

In the current study, we used qRT-PCR as a third method to validate the predictive value of TSC22D1 and PSAP in tamoxifen therapy. The patient cohort in this study is clinically relevant because it included a large group of patients with stable disease. We investigated whether the expression levels of TSC22D1 and PSAP in primary breast tumors

A:



B:

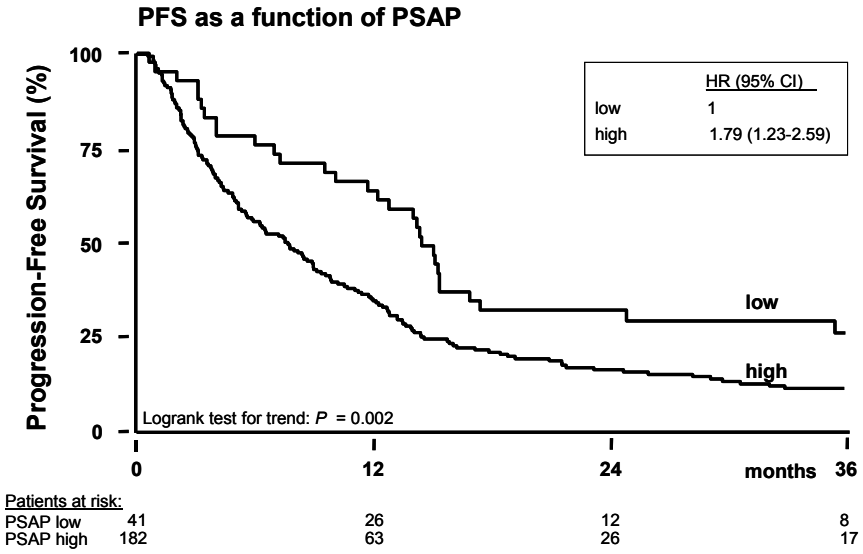


Figure 4.1 Kaplan-Meier curves of progression-free survival for advanced breast cancer patients after start of first-line tamoxifen therapy as a function of TSC22D1 (A) and PSAP (B) mRNA levels. On the basis of cutpoint analyses, patients were divided into two groups, having low or high mRNA levels. Patients at risk are indicated.

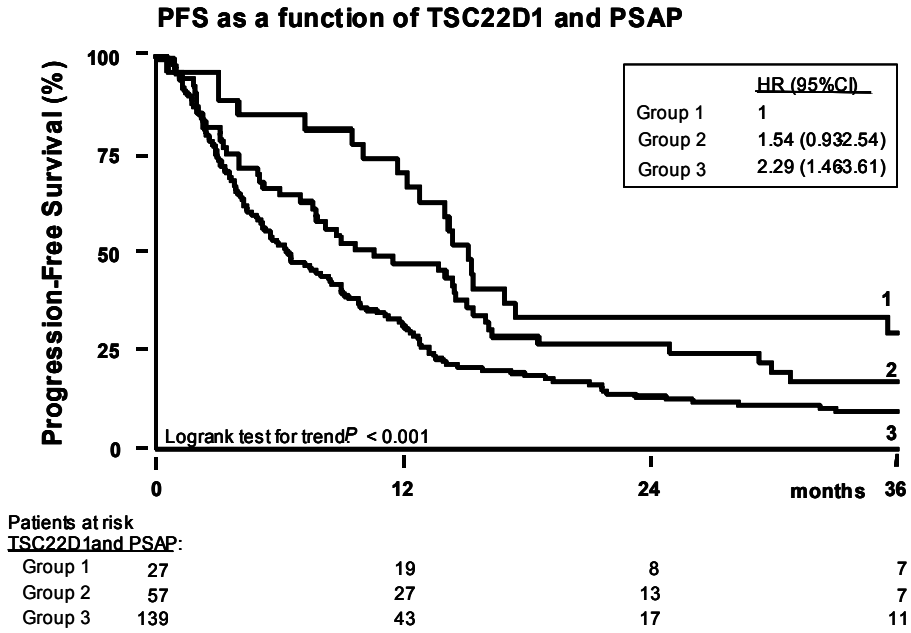


Figure 4.2 Kaplan-Meier curve of progression-free survival for advanced breast cancer patients after start of first-line tamoxifen therapy as a function of combined TSC22D1 and PSAP mRNA levels. Patients were divided into three groups having low levels for both genes (group 1), low levels for TSC22D1 or PSAP (group 2), and high levels for both genes (group 3). Patients at risk are indicated.

could predict the outcome of first-line tamoxifen treatment in patients with advanced disease. Our results confirm that patients having high mRNA levels of TSC22D1 and/or PSAP have a shorter PFS compared with patients having low levels of these genes.

TSC22D1 encodes a leucine-zipper transcription factor and belongs to the large family of early response genes. It was originally isolated as a Transforming Growth Factor, beta-1 (TGFβ) inducible gene in mouse osteoblasts (13). TSC22D1 was proposed as a tumor-suppressor gene because it participates in growth inhibition of brain tumors, salivary-gland tumors and prostate cancers (14-16). TSC22D1 was found to be downregulated in the presence of estrogens in MCF7 breast cancer cells (17) which was in concordance with our Spearman rank correlation data. Expression of the gene was shown to be up-regulated through progesterone in growth-inhibited breast cancer cells (18).

With regard to TGFβ signaling it was shown that TSC22D1 enhanced TGFβ signaling in U973 cells by interaction with SMAD4 (19). Furthermore, TSC22D1 increased levels of Cyclin-dependent Kinase Inhibitor 1A (CDKN1A, also known as p21 or CIP1), a downstream component of TGFβ, in colon carcinoma cells (20). In addition, TGFβ is a well known inhibitor of cell proliferation, which can be activated by the presence of tamoxifen (21). These data support an inhibitory effect of TSC22D1 on tumor proliferation and seem to be in contrast with the fact that high levels of TSC22D1 are associated with tamoxifen resistance. However, it has been described that TGFβ can turn into a promoter of progres-

sion in later tumor stages and can stimulate tumor angiogenesis, extracellular matrix degradation, inhibition of antitumor immune response, and epithelial-to-mesenchymal transition (22).

PSAP is a lysosomal precursor protein of the saposins A, B, C, and D. PSAP has been reported to be present in conditioned media from the ER negative MDA-MB-231 as well as the ER positive MCF-7 breast cancer cell lines and in SV40 transformed normal HBL100 breast cells. Besides that, it also exists uncleaved in many biological fluids, including human milk (23, 24). Estrogens have been reported to stimulate PSAP production in MCF-7 cells. Moreover, PSAP interacts with procathepsin D in human breast cancer cells (25, 26), suggesting a role in tumor invasiveness and metastasis. Misasi *et al*. have demonstrated an anti-apoptotic effect of PSAP in neuronal as well as non-neuronal cells (27, 28). They have shown that PSAP activates Mitogen-activated Protein Kinase 1 and 3 (ERK-2 and -1) and spingosine kinase, and they propose a possible role in cell survival for the MAPK and PI3K-Akt pathways through which these genes exert their function. Interestingly, both pathways have previously been reported to be associated with tamoxifen resistance (29, 30). The latter supports our observed association of high levels of PSAP with tamoxifen treatment failure.

Almost two-thirds of the patients show high expression levels of both PSAP and TSC22D1. This group has an even poorer treatment outcome. The relative high percentage of patients with high levels of both genes may be explained by CDKN1A. It has been reported that TSC22D1 increase CDKN1A levels. Besides its well-known tumor-suppressive effects, high levels of CDKN1A have also been reported to have an unfavorable effect in anticancer treatment (31). In permanently growth-inhibited tumor cells, a state that bears resemblance to tamoxifen treatment, CDKN1A has been shown to induce genes having an anti-apoptotic or mitogenic activity, including PSAP (32, 33). Furthermore, this indicates that upregulation of CDKN1A also supports the possible tumor-promoting capabilities of TSC22D1.

In the present study, qRT-PCR analysis of mRNA levels of TSC22D1 and PSAP in primary breast-cancer specimens validates our previous genome wide and *in vitro* findings of these genes being associated with tamoxifen resistance. Since the mechanisms of action of PSAP and TSC22D1 have not been well characterized, the biological processes involved in tamoxifen resistance remain to be elucidated. As discussed above, several lines of evidence have been reported already. It would be intriguing though to unravel the precise role of these genes in resistance with respect to the clinical significance in breast cancer.

We showed an association of high expression levels for both genes with failure of tamoxifen treatment in patients with recurrent breast cancer. On the basis of our functional screen, both genes have a putative causative role in therapy resistance and this

may give new perspectives on biological pathways containing new drugable targets for tamoxifen therapy.

ACKNOWLEDGEMENTS

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CHAPTER 5

CHARACTERIZATION OF BCAR4, A NOVEL GENE CAUSING TAMOXIFEN RESISTANCE IN HUMAN BREAST CANCER CELLS

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To be submitted

ABSTRACT

Breast Cancer Antiestrogen Resistance gene 4 (BCAR4) has been identified in a functional screen as a gene causing tamoxifen resistance in human breast cancer cells. Here we describe further characterization of this novel gene. Ectopic expression of BCAR4 in ZR-75-1 resulted in cell proliferation both in the absence of estrogen and in the presence of various antiestrogens. The gene also caused ICI182,780-resistant proliferation of MCF7 cells when over-expressed. Two different conserved primate homologues (rhesus monkey and African green monkey) induced tamoxifen-resistant cell proliferation when transfected to ZR-75-1 cells as well. Using fusion proteins of BCAR4 with FLAG-tags the protein was localized to the cytoplasm and to a lesser extent to the cell membranes. Injection of ZR-75-1 cells expressing BCAR4 into nude mice resulted in rapidly growing tumors, whereas the parental cell line was unable to do so. Proliferation assays in the presence of small-molecule inhibitors indicated a possible role for mitogen-activated protein kinase 14 (p38) in BCAR4 signaling. In conclusion, BCAR4 is a strong transforming gene which causes estrogen independence and induces solid tumors in nude mice. These results indicate that the signaling pathway that is activated by BCAR4 may play a role in breast cancer progression.

INTRODUCTION

Breast cancer is the most frequent malignancy in women in the western world. It has been well established that the hormone estrogen plays a significant role in breast-cancer development and progression. Selective estrogen receptor (ER) modulators, like tamoxifen, have been the most commonly used hormonal therapy for ER-positive breast cancer during the last three decades. However, resistance to this therapy presents a major challenge in disease management and are the subject of active investigation.

To identify genes involved in tamoxifen resistance, we have used retroviral transduction of cDNA libraries into the ZR-75-1 human breast cancer cell line (1). With this approach, we have identified 73 genes with a possible role in tamoxifen resistance. After application of stringent selection criteria, 7 genes were stated to have a causative role in resistance. Among this set were Epidermal Growth Factor Receptor (EGFR) and Neuregulin 1 (NRG1), which already have been shown to be related with tamoxifen resistance (2-4). In addition, we identified a novel gene designated as BCAR4. Ectopic expression of BCAR4 induced tamoxifen-resistant proliferation in the tamoxifen-sensitive ZR-75-1 breast cancer cell line. It also engenders anchorage-independent proliferation in the absence of estrogens, while the parental cells are unable to do so (1). Analysis of our array data (5) and published array data (6, 7) indicated that BCAR4 expression is present in approximately 10% of breast cancers. These observations prompted us to further characterize this gene for elucidating its relevance in endocrine resistance.

MATERIALS AND METHODS

Cell lines and culture conditions

Cell culture was performed as described previously (2). ZR-75-1 (8) cells were cultured in RPMI 1640 medium (Invitrogen, Breda, the Netherlands) supplemented with 10% heat-inactivated bovine calf serum (standard medium) (Hyclone, Logan, UT) and 1 nM 17β-estradiol (E_2) (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands). MCF7 and COS-1 cell lines were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Sigma-Aldrich Chemie).

Generation of constructs and cell lines

FLAG tags with amino acid sequence N-DYKDDDDK-C were fused to BCAR4 via polymerase chain reaction. The N-terminal tag, starting with a Kozak sequence, was fused in-frame to the BCAR4 protein. Hence, the original initiator methionine of BCAR4 was not retained in this construct. The C-terminal tag, including a stop codon, was fused to the end of the coding sequence. ZR-75-1 cells containing FLAG-tagged BCAR4 constructs

were generated using retroviral infection as described before (1). In brief, DNA of FLAG-tagged BCAR4 constucts were cloned into the LZRS-IRES-Neo expression vector (9) and viral particles were produced. ZR-75-1 cells were infected with these particles and after selection with G418 (Invitrogen), pools of construct-containing cells were obtained. Previously generated ZR/BCAR4 (wt) and ZR/LZRS cells were used as controls (1).

Monkey-BCAR4 DNA fragments were retrieved from rhesus-monkey genomic DNA, isolated from blood, and DNA of the COS-1 cell line (African green monkey) using PCR and BCAR4-specific primers (forward: TCACCATGTACCAACCTATCC, reverse: ACAATGCTCAGGAGACTTAG, nested forward: CCATGTACCAACCTATCCAAAC, nested reverse 1: CAGGAGACTTAGTTCCAAAGACG, nested reverse 2: AGACGAAGATGCCAGGGTTC). PCR fragments were cloned into the pCR 2.1-TOPO vector (Invitrogen), sequenced, and subsequently transferred into the LZRS-IRES-Neo expression vector. The expression constructs were transfected into ZR-75-1 cells, in duplicate, using FuGENE 6 (Roche Diagnostics, Almere, The Netherlands). After selection with G418 (Invitrogen) resistant colonies were pooled. We used cells containing empty vectors as controls.

Proliferation assays

Pools of ZR/BCAR4, ZR/LZRS, MCF7/BCAR4, and MCF7/LZRS cells were harvested by trypsinization and counted with a Coulter Z1 cell counter (Coulter Electronics Ltd, Luton, UK). Subsequently, $3.5x10^5$ cells were plated in triplicate in 25 cm^2 tissue culture flasks (Corning, Schiphol, The Netherlands). Experimental medium containing 100nM ICI182,780 (Zeneca, Pharmaceuticals, Macclesfield, UK) was changed twice a week. Cells were trypsinized, counted and replated in fresh medium at the initial density once a week.

Pools of ZR/BCAR4 N-terminal FLAG-tagged and C-terminal FLAG-tagged cells were trypsinized and counted with a counting chamber. $7x10^5$ cells were plated in 25 cm^2 tissue culture flasks (Corning) in triplicate. Experimental medium containing 1μM 4-hydroxytamoxifen (OH-TAM) (Sigma-Aldrich Chemie) was changed twice a week. Cells were trypsinized, counted and replated in fresh medium at the initial density at days 10, 21, and 30.

ZR/BCAR4 and ZR/LZRS control cells were seeded in 96-wells plates at a density of 5000 cells/well in a final volume of 200μl. The media contained increasing concentrations of OH-TAM (from 100pM to 10μM), raloxifene hydrochloride (Sigma-Aldrich Chemie) (from 100pM to 10μM), E_2 (from 1pM to 10 nM) and BCS (from 0.1% to 20%) or 1% ethanol or 0.1% DMSO as vehicle controls. For each concentration and time point cells were plated in six fold. After 4 and 6 days of culture at 37˚C 10μl of the proliferation reagent WST-1 (Roche Diagnostics) were added to each well, and the cells were returned to the

incubator for one hour. Absorbance was determined at 405 nm with a Multiskan Ascent microplate photometer (Thermo Fisher Scientific, Breda, The Netherlands).

Pools of ZR-75-1 cells with rhesus monkey BCAR4 (ZR/RM) or African green monkey BCAR4 (ZR/AGM) expression constructs were harvested and plated in fifteen 25 cm^2 tissue culture flasks (Corning) with standard medium containing 1μM OH-TAM at a density of $1.5x10^5$ cells/flask. ZR-75-1 cells with empty vectors were used as controls. At days 4, 7, 11, and 14 the cells were trypsinized and counted using a Coulter Z1 cell counter (Coulter Electronics Ltd) in triplicate.

ZR-75-1 cells and the derived transfectants containing BCAR1 (10), BCAR3 (11), EGFR (2) and BCAR4 (1) were seeded into 96 wells plates at a density of 1000 to 2500 cells per well in standard medium. After two days, serial dilutions of drugs inhibiting EGFR (AG1478 and PKI166), MEK (U0126), MAPK14 (SB203580), SRC (PP2), and PI3K (LY29400) were added. Cultures were supplemented with E_2, OH-TAM or EGF in final concentrations of 1nM, 1μM and 10 ng/ml respectively. After five days, MTT reagent (5 mg/ml in PBS) was added and incubated for maximally two hours at 37°C as described previously (2) . Formazan production was measured using a Multiskan Ascent microplate photometer. Absorbance was corrected for background, averaged and normalized to the control cultures. All drug concentrations were tested at least in triplicate wells and the assays were performed in duplicate (replicated at least once). IC50 values were calculated from the dose response curves. For comparison, the lowest IC50 value for a particular drug was set at 1.0 and used to assess the relative sensitivities of the different cell lines and culture conditions.

Cellular localization of BCAR4

ZR-75-1 cell lines expressing FLAG-tagged BCAR4 proteins were cultured on LAB-TEC microscope slides (Nalge-Hunc Int., Rochester, NY, USA) and fixed with methanol at -20°C. Protein localization was determined by light microscopy and immunofluoresence using anti-FLAG M2 (Amersham Biosciences Benelux, Roosendaal, The Netherlands) and Cy3-labeled goat anti-mouse (Jackson Immuno Research Labs, Suffolk, UK) antibodies. For detection of FLAG-tagged BCAR4 proteins on western blots we also used Anti-FLAG M2 (Amersham Biosciences Benelux) as primary antibody and HRP-conjugated anti-mouse Ig (Amersham Biosciences Benelux).

Xenograft formation in nude mice

ZR/BCAR4 and ZR/LZRS cells were harvested and resuspended in fresh culture medium and matrigel (5:1) (Collaborative Research, Bedford, MA, USA). Cell suspensions were subcutaneously injected ($2x10^6$ cells /site) under the mammary fat pad region of eight

6-week old female *nu/nu* nude mice, at the right and left 4th nipple. The developing tumors were measured twice weekly.

RESULTS

Ectopic expression of BCAR4 induces resistance to the pure antiestrogen ICI182,780 in ZR-75-1 and MCF7 breast cancer cells.

Previously we showed, that ectopic expression of BCAR4 enables ZR-75-1 cells to proliferate in the presence of OH-TAM (1). To further establish the role of BCAR4 in antiestrogen resistance, we investigated whether the same result could be obtained in a different estrogen-dependent cell line, MCF7, and in the presence of the pure antiestrogen 100nM of ICI182,780. We generated MCF7/BCAR4 cells and tested these, together with ZR/BCAR4 cells, for proliferation in the presence of ICI182,780. As shown in Figure 5.1A, ZR/BCAR4 cells were fully resistant to this pure antiestrogen. Similarly, MCF7/BCAR4 cells were able to proliferate in the presence of ICI182,780 while vector-control cells were growth inhibited (Figure 5.1B). From these results it is shown that ectopic expression of BCAR4 transforms both breast cancer models from an antiestrogen-sensitive to an antiestrogen-resistant state.

Proliferation of BCAR4 cells is dependent on serum but not on estradiol.

We compared the ability of ZR-75-1 and ZR/BCAR4 cells to proliferate in medium containing different concentrations of bovine calf serum (BCS), 17β-estradiol (E_2), and the

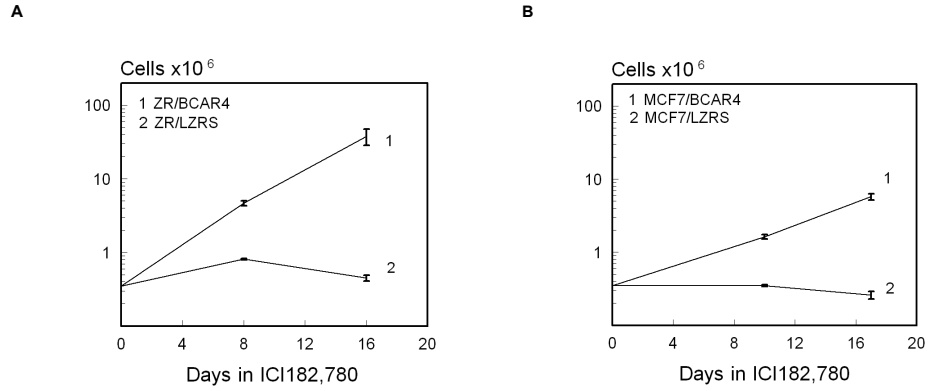

Figure 5.1

BCAR4 induces resistance to the pure antiestrogen ICI182,780 in ZR-75-1 (A) and MCF7 (B) cell lines. Cells containing BCAR4 and vector control cells were cultured in medium containing 100nM of ICI182,780. At the timepoints indicated the cells were counted and replated at the initial density. Cumulative cell numbers and SDs are presented.

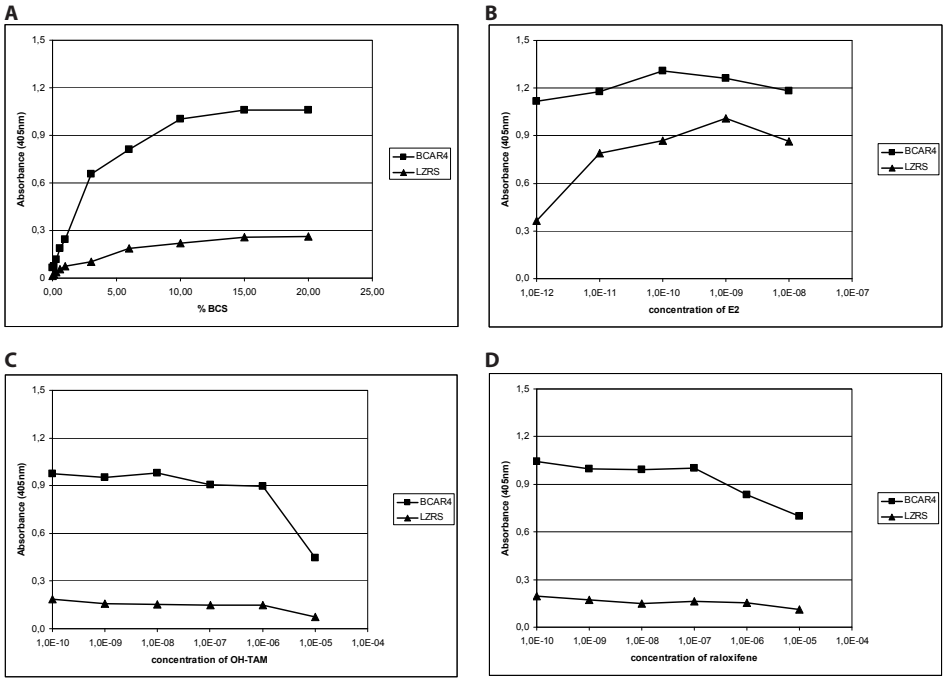

Figure 5.2

Dose-dependent effect of various factors on the proliferation of ZR/BCAR4 cells. ZR/BCAR4 and ZR/LZRS cells seeded in 96-well plates were cultured with various concentrations of BCS, E2, OH-TAM, and raloxifene. Cell viability was determined using the WST-1 assay on day 6. Proliferation of ZR/BCAR4 cells is dependent on serum components (A). No dose-dependent effects on BCAR4 cell proliferation were seen after addition of E2 (B) (whereas proliferation of the vector control cells is strongly stimulated by E2), OH-TAM (up to 1 μM) (C) or raloxifene (up to 10μM) (D). Similar curves were obtained at day 4.

antiestrogens OH-TAM and raloxifene. As shown in Figure 5.2A, the BCAR4 cells remained dependent on BCS for proliferation. In contrast, addition of E_2 up to 100pM caused only marginal growth stimulation in ZR/BCAR4 cells, while proliferation of ZR-75-1 cells strongly depended on the addition of E_2 (Figure 5.2B). No significant growth inhibition of ZR/BCAR4 cells was obtained by addition of OH-TAM up to 1μM (Figure 5.2C) or raloxifene up to 100nM (Figure 5.2D). Inhibition of proliferation at higher concentrations may be due to toxicity of the drugs. No significant alteration of growth of both cell lines was observed by the addition of vehicle (ethanol or DMSO at a final concentration of 1% and 0.1% respectively) alone.

BCAR4 is highly conserved in primates, but not in rodents

A highly conserved homologous BCAR4 gene is found only in primates among the genomes presently sequenced. BLAST searches revealed a 100% similarity between human and chimpanzee BCAR4 and a 93% similarity between human and rhesus monkey BCAR4. BLAST searches failed to identify a conserved BCAR4 homologue in other species including mouse and rat. To compare the function of two primate homologues with

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                                                                    --
BCAR4Hum  MYQPIQTYPWMNLSRRREFRCLSCSECLLVTCLGLSTVILGLIVVLQDPSDSVVFSTGLT 60
Rhesus    ..........S..G.Q.........................V.................
AGM       ..........S..G.Q.........................V.................

          -----------------
BCAR4Hum  MIAIGAFFVVLTGVTALCTVTVDENLQKTTRLRLGVIRKSGSLQGTTEPSMTHSIIASTSL 121
Rhesus    ........I.........................M...........A..T...V......
AGM       ........I...................M.................A......V......
```

Figure 5.3

Alignment of primate BCAR4 proteins. The human BCAR4 protein is 100% identical to chimpanzee BCAR4 protein (acc nr XM_510822). Rhesus monkey and African green monkey (AGM) sequences were aligned with the Clustal W multiple sequence alignment software tool. Amino acid identities (93%) are shown as dots. The positions of the signal peptide (a.a. 1-41, solid line) predicted using the SignalP 3.0 Server, and of the transmembrane domains (a.a. 27-46 and 59-77, dashed line) as predicted by TMpred are shown above the sequence.

the human BCAR4, we retrieved the BCAR4 sequences from rhesus monkey and African green monkey DNA using PCR and generated expression constructs. BCAR4 protein sequences of rhesus monkey and African green monkey were closely related to each other and showed 93% similarity to the human protein (Figure 5.3). Proliferation assays showed that ZR-75-1 cells containing expression constructs with BCAR4 derived from rhesus monkey (Figure 5.4A) and African green monkey (Figure 5.4B) were also able to proliferate in the presence of OH-TAM.

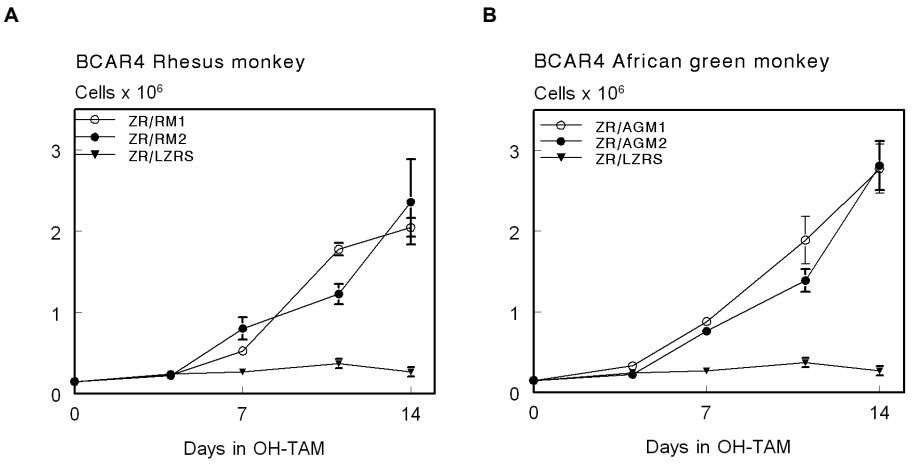

Figure 5.4

Primate homologues of BCAR4 induce OH-TAM-resistant proliferation. Pools of ZR-75-1 cells containing expression constructs with rhesus monkey and African green monkey BCAR4 (ZR/RM and ZR/AGM respectively) were plated in 15 culture flasks with medium containing OH-TAM. At the timepoints indicated cells were counted in triplicate. Cell numbers and SDs are presented. A. proliferation curves of cells containing rhesus monkey BCAR4. B. proliferation curves of cells containing African green monkey BCAR4.

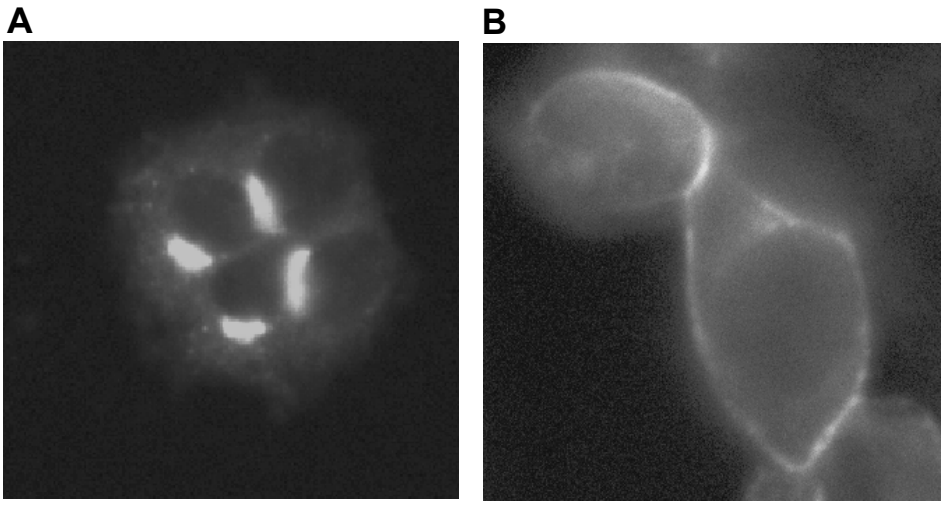

Figure 5.5
Subcellular localization of FLAG-tagged BCAR4 proteins. ZR-75-1 cell lines expressing FLAG-tagged BCAR4 proteins were cultured on microscope slides, fixed, and labeled with primary anti-FLAG and secondary Cy3-labeled antibodies. Fluorescence light microscopy revealed localization of C-terminal (3') FLAG-tagged BCAR4 protein in the cytoplasm (A). N-terminal (5') FLAG-tagged protein was predominantly localized to the cell membranes (B).

Subcellular localization of BCAR4

To elucidate possible functions of BCAR4, we examined the subcellular localization of the BCAR4 protein. A fusion protein was generated consisting of the BCAR4 polypeptide and a FLAG-tag at the N-terminal or C-terminal sites. ZR-75-1 cell lines expressing FLAG-tagged BCAR4 proteins were labeled with appropriate primary and secondary antibodies. Fluorescence light microscopy revealed that the C-terminal FLAG-tagged BCAR4 protein was localized to the cytoplasm, possibly to the Golgi apparatus and to the endoplasmatic reticulum, and to a lesser extend to the secretory vesicles and the cell membranes (Figure 5.5A). The N-terminal FLAG-tagged BCAR4 protein was localized to the cell membranes and to a lesser extent to the cytoplasm (Figure 5.5B). In control cell cultures no fluorescent signal was observed. Production of the N-terminal and C-terminal FLAG-tagged BCAR4 proteins was confirmed by immunoprecipitation with antibodies directed against the FLAG tag and western blot analysis (Figure 5.6).

To test whether the fusion of the FLAG-tags to BCAR4 influenced the proliferation capacity of the cells in the presence of OH-TAM, growth curves were generated. The cell line expressing N-terminal FLAG-tagged BCAR4 protein was able to proliferate efficiently in the presence of OH-TAM, but the proliferation rate of cells with the C-terminal FLAG-tagged BCAR4 protein appeared reduced as compared to the wild-type BCAR4 (Figure 5.7)

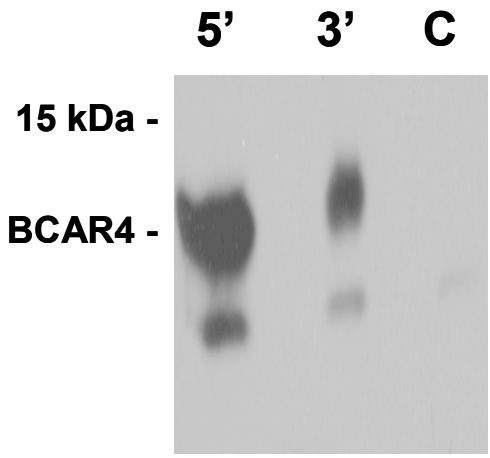

Figure 5.6
Detection of FLAG-tagged BCAR4 proteins. Western blot analysis showed production of C-terminal (3’) and N-terminal (5’) FLAG- tagged proteins. Cells containing an empty expression vector were used as control (C).

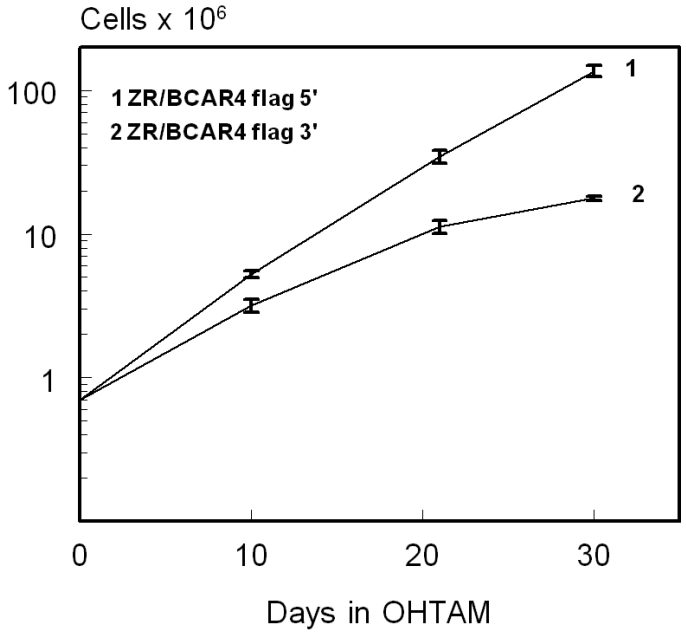

Figure 5.7
FLAG-tagged BCAR4 proteins are still able to transform ZR-75-1 cells to an antiestrogen-resistant phenotype. Cells with BCAR4 protein fused to a FLAG-tag at the C-terminal (3’) or N-terminal (5’) ends were cultured in medium containing OH-TAM. At the timepoints indicated the cells were counted and replated at the initial density. Cumulative cell numbers and SDs are presented. Proliferation of cells containing C-terminal FLAG-tagged BCAR4 protein was reduced.

BCAR4 promotes xenograft tumor formation in nude mice

Previously we determined that over-expression of BCAR4 induces anchorage-independent transformation of ZR-75-1 cells (1). Therefore, we speculated that this gene might be able to induce tumor formation in nude mice. To test their tumorigenicity, $2x10^6$ empty vector containing ZR/LZRS control cells, and ZR/BCAR4 cells were injected into the mammary fat pads of the fourth right and left nipple of female mice. Within two weeks, three out of five mice with ZR/BCAR4 xenografts developed tumors on both sides (Figure 5.8A). One out of five mice had tumor formation on only one side and in one mouse no tumors were detected within 6 months. ZR/LZRS injected mice failed to develop any tumors over a period of 6 months follow-up (Figure 5.8B). In the presence of an estrogen release pellet, injection of both ZR/LZRS and ZR/BCAR4 cells caused tumor formation in these mice (data not shown). These results show, that ZR/BCAR4 cells acquired the capability to drive tumor formation without addition of estrogen.

BCAR4 and the MAPK pathway

Comparing cell proliferation with other tamoxifen-resistant variants of ZR-75-1 cells assessed the sensitivity of the BCAR4 cells to several types of intracellular-signaling inhibitors. The effects of the drugs on proliferation capacity were measured with MTT under normal culture conditions (estrogen supplemented) and under selective conditions (in the presence of the anti-estrogen OH-TAM). The most prominent inhibitory effects were noted in the EGFR transfected cells when stimulated with its ligand EGF (Table 5.1). BCAR4 cells showed high sensitivity for the MAPK14 (p38) inhibitor under estrogen

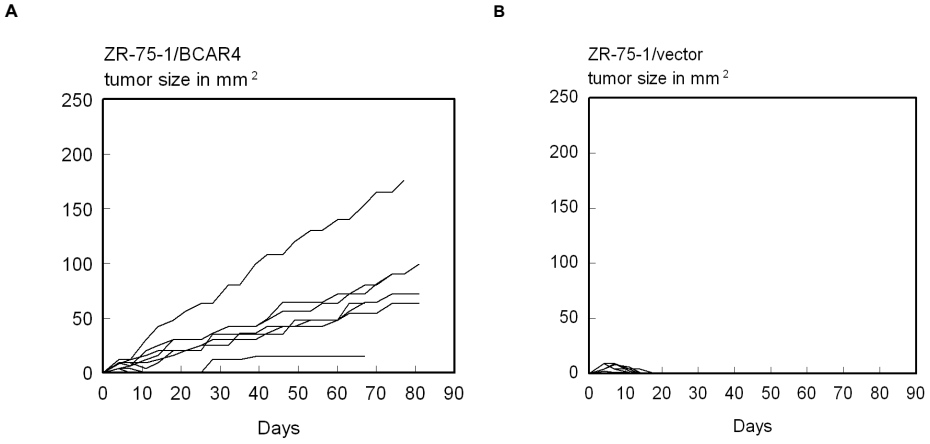

Figure 5.8

ZR/BCAR4 cells induce tumor formation in nude mice. The mice were injected with ZR/BCAR4 cells or ZR/LZRS control cells in the mammary fat pad of the fourth left and right nipples. A. Four out of five mice injected with ZR/BCAR4 cells developed rapidly growing tumors. B. None of the mice injected with ZR/LZRS cells showed any tumor formation.

Table 5.1 Relative Sensitivity to small molecule inhibitors#.

Target	Drug	ZR-75-1 E2	BCAR1 E2	BCAR1 OH-T	BCAR3 E2	BCAR3 OH-T	BCAR4 E2	BCAR4 OH-T	EGFR E2	EGFR OH-T/ EGF
PI3K	LY294002	1.8	1.5	1.5	1.5	1.0	3.0	3.8	2.6	7.5
SRC	PP2	5.5	3.8	2.7	2.4	1.0	5.0	7.2	4.7	7.3
MAPK14	SB203580	4.3	5.6	5.0	4.0	1.9	2.1	1.0	4.3	1.4
MEK	U0126	ND	1.0	1.0	1.0	1.8	1.8	1.8	1.0	7.9
EGFR	AG1478	ND	18.5	18.5	18.5	18.5	13.0	12.0	26.5	1.0

Average (at least two independent experiments) sensitivities of cell lines for a particular drug are given relative to the most sensitive cell line IC50 = 1.0. ND= not determined.

conditions and were even more sensitive under OH-TAM conditions. In contrast, inhibition of PI3K or SRC was least effective on proliferation of BCAR4 cells, although the differences are subtle.

DISCUSSION

BCAR4 has previously been identified in a functional screen for genes responsible for tamoxifen resistance. We showed that BCAR4 causes tamoxifen resistance in ZR-75-1 cells, and that cells expressing the gene were transformed to an anchorage-independent phenotype (1). In this study, we demonstrated that ectopic expression of BCAR4 also supports proliferation in the presence of the pure antiestrogen ICI182,780 (Faslodex/Fulvestrant) and raloxifene, and in another estrogen-dependent human breast cancer cell line, MCF7. These results indicate that the cells are no longer dependent on estrogens for their proliferation, in contrast to the parental cell line. However, the BCAR4 cell line still required components present in the bovine serum BCS for proliferation.

The amino acid sequence of BCAR4 does not reveal conserved protein domains, with the only exception of a putative anchor signal and two transmembrane domains, indicating that the protein might be localized to cell membranes. These features have been retained in the rhesus and African green monkey BCAR4 peptides (http://www.ch.embnet.org/software/TMPRED_form.html, http://www.cbs.dtu.dk/services/SignalP/). To explore the cellular localization of BCAR4, we generated BCAR4-FLAG-tagged fusion proteins. We investigated if ZR-75-1 cells, stably expressing these fusion proteins were still able to proliferate in the presence of OH-TAM. Although tags have been added to

the BCAR4 protein, the cells still have an antiestrogen-resistant phenotype. However, the proliferation of cells containing the C-terminal FLAG-tagged protein appeared reduced, as compared to the wild-type BCAR4, suggesting functional interference. Fluorescence light microscopy revealed that the fusion proteins were localized in the cytoplasm and the cell membranes. Localization to the cell membranes was predominantly observed with the N-terminal FLAG-tagged construct and in lesser extent with the other tagged construct. These results are supported by the presence of the putative signal anchor and transmembrane domains. Cells expressing the C-terminal FLAG-tagged protein showed an accumulation of BCAR4 protein in the cytoplasm, possibly in the Golgi apparatus and the endoplasmatic reticulum. However, the presence of the transmembrane domains suggests that the protein may be localized on the cell membrane instead of the cytoplasm. This and the reduced growth advantage of the cells expressing the C-terminal FLAG-tagged protein in the presence of tamoxifen indicate that the protein accumulation in the cytoplasm might be artificial due to disturbance of the protein structure at the C-terminal side. It has previously been reported that addition of a tag to the C-terminus of a protein may alter its subcellular localization in comparison to the native protein (12). To actually visualize the localization of the original protein without a tag, BCAR4 specific antibodies are needed and must be generated.

The BCAR4 protein has well conserved homologues in the chimpanzee (100% identical), the rhesus monkey (93% identical), and the African green monkey (93% identical). Proliferation assays showed that these homologues are still able to transform ZR-75-1 cells into an OH-TAM resistant phenotype. The absence of the gene in non-primate species may indicate that BCAR4 has emerged as a new gene during the primate evolution. Johnson *et al*. reported that new gene families may have been positively selected during the emergence of humans and African apes and that a small fraction of human genes may not possess orthologues in the genomes of model organisms, such as the mouse (13). Although primate-specific genes are rare, some have been identified. For example, analyses of the chromosome 21 gene content revealed a small proportion of (possible) primate-specific genes (14, 15). Furthermore, the primate-specific Ku86 Autoantigen Related Protein-1 (KARP-1) and Coordinated Expression to IRXA2 (CEI) genes have been identified (16, 17).

To explore whether BCAR4 is able to form tumors *in vivo*, female nude mice were injected in the mammary fat pad with BCAR4 expressing cells. Eighty percent of these mice developed rapidly growing tumors versus none of the control mice. The ZR/LZRS control cells only induced tumor development in the presence of an estrogen-release pellet. These results show that BCAR4 induces tumor growth *in vivo* independent of additionally administered estrogens.

In recent years, many small-molecule inhibitors of signaling components have been identified and used for research purposes and for clinical applications (18-20). Our pro-

liferation assays with small-molecule inhibitors indicated that MAPK14 (p38) might play a role in BCAR4 signaling, because ZR/BCAR4 cells show a relatively higher sensitivity to the MAPK14 inhibitor. However, we have to remain cautious in drawing conclusions, because many small-molecule inhibitors exhibit cross reactivity with other related (and probably non-related) target molecules (21). In order to deduce unknown signaling routes from the cell-growth inhibitory effects of these drugs, adequate controls are essential. Independent validation of our results using an independent inhibitor against MAPK14 may shed some light on the actual contribution of MAPK14 in BCAR4 signaling.

In conclusion, BCAR4 is a transforming gene with oncogenic potential that makes breast cancer cells independent of estrogen stimulation. Our data suggest that BCAR4 signaling activates the MAPK pathway which has been demonstrated to be involved in antiestrogen resistance before (1, 22). CDNA array comparative genomic hybridization (CGH) on a panel of breast tumors revealed amplification of the 16p13 region containing BCAR4 (23). Gene amplification is one of the mechanisms underlying activation of oncogenes (24), and has previously been associated with ERBB2-mediated antiestrogen resistance (25). Thus, amplification and/or over-expression of BCAR4 might stimulate antiestrogen-resistant tumor progression in breast cancer patients. SAGE data (http//bioinfo.amc.uva.nl/HTMseq/), EST profiles (www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Hs.24611), and different sets of array data (5-7) have shown so far that BCAR4 is only expressed in normal human placenta and embryonic tissue, and in breast cancer. Therefore targeting this gene would specifically affect the tumors expressing it, indicating the potential clinical value of this gene in breast cancer therapy.

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CHAPTER 6

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Tamoxifen is the most extensively used drug in the treatment of breast cancer. It has been used in the clinic during the last four decades and has shown to be successful in prevention of breast cancer, as adjuvant therapy, and as a treatment of metastatic disease (1, 2). Tamoxifen has shown the ability to reduce recurrences of the cancer and to prolong life. However, despite the success of tamoxifen therapy, the patients eventually develop resistance to the drug, resulting in breast cancer progression. To overcome tamoxifen resistance or to develop new (patient-tailored) treatment strategies, it is necessary to understand the underlying mechanisms. The aim of the experimental work described in this thesis was to identify genes involved in tamoxifen resistance.

RETROVIRAL TRANSDUCTION OF CDNA LIBRARIES

For identifying genes responsible for tamoxifen resistance in human breast cancer we used retroviral transduction of cDNA libraries in ZR-75-1 cells. Unlike profiling approaches like microarray analysis and serial analysis of gene expression (SAGE), this technique allows the establishment of causal relationships between gene overexpression and tamoxifen resistance, rather than just correlation. As described in chapter 2, we uncovered 7 genes to cause tamoxifen resistance in the ZR-75-1 breast cancer cell line. Furthermore, 18 genes were assigned as candidates and would require further validation to establish their role in tamoxifen resistance. From these experiments, it is clear that inhibition of cell proliferation in the presence of tamoxifen may be deregulated by enhanced expression of multiple different genes.

Introduction of cDNA libraries may result in overexpression of the genes corresponding to the cDNAs inserted in the target cells. Biological effects of loss-of-gene function are generally not detected using this strategy. The consequences of gene loss may be assessed by RNA interference (RNAi) which has been employed to knock down specific transcripts (3, 4). Similar to the cDNA libraries, RNAi libraries can be introduced into the cells to perform large-scale loss-of-function screens. Several genome-wide RNAi libraries in different vector systems (5) have become available, but there are still technical challenges to overcome. Short hairpin RNA (shRNA) and microRNA-adapted short hairpin RNA (shRNAmir) processing is rate limited because of their endogenous processing by Drosha and Dicer resulting in partial knockdown levels. This may also be a favorable situation because they may have potentially less concentration-dependent off-target effects as seen with short interfering RNAs (siRNAs) (5). However, off-target effects cannot be ruled out and have to be considered. Furthermore, large-scale RNAi screens may produce a lot of false positives (6). Despite these hurdles, knock-down strategies may be useful to obtain further insight into the mechanisms of tamoxifen resistance.

GENES ASSOCIATED WITH TAMOXIFEN-THERAPY FAILURE IN BREAST CANCER PATIENTS

FGF17 was identified as one of the genes having a causative role in tamoxifen resistance of ZR-75-1 breast cancer cells (chapter 2). To assess whether this gene or the FGF receptors (FGFR) 1, 2, 3 and 4 are associated with clinical outcome of tamoxifen therapy in breast cancer patients, mRNA levels of these genes were measured in primary breast tumors using quantitative RT-PCR. For all patients extensive clinical follow-up was available. Chapter 3 describes a clinical association of FGFR4 with tamoxifen resistance in patients with metastatic breast cancer. High levels of FGFR4 mRNA predict a poor therapy outcome. However, no association between FGF17 and tamoxifen therapy was observed.

FGF17 was recovered from retroviral transduction experiments with a human brain tissue cDNA library (chapter 2). EST expression profiling and SAGE analysis showed that FGF17 is predominantly expressed in embryonic and brain tissue. This indicates that the type of genes recovered from these kind of experiments can strongly depend on the tissue source, gene expression levels and the quality of the cDNA library. The absence of FGF17 in breast (cancer) tissue may explain its lack of association with failure of tamoxifen therapy. However, it is strongly suggested that the pathway activated by FGF17 may play an important role in resistance. FGF17 was recently found to mainly activate FGFR4 in epithelial lineages (7), supporting the theory that activation of FGFR4 is responsible for causing tamoxifen resistance in ZR-75-1 breast cancer cells. FGF17 forms a subfamily with FGF8 and FGF18 (8). Expression levels of FGF8 were shown to be higher in breast cancer than in normal breast tissue (9). It would therefore be interesting to assess whether FGF8 has a predictive value for the type of response to tamoxifen treatment.

In our quantitative RT-PCR experiments we did not take into account the splice variants of the genes involved, like of FGFR1, 2, and 3. Splice variants may have different effects on therapy outcome and this has to be considered in future research. Similarly, particular SNPs may play a role in gene activity. Therefore studying SNPs in de genes identified may provide insight into the mechanisms resulting in overexpression of these genes.

BCAR4 A NOVEL GENE CAUSING TAMOXIFEN RESISTANCE

Retroviral transduction of a human placenta cDNA library into ZR-75-1 breast cancer cells revealed, amongst others, a novel gene designated BCAR4 (chapter 2). Very little was known about this gene as it had no homologues in non-primates or typically conserved protein domains. Only a signal anchor and two transmembrane regions were

predicted in the protein sequence. SAGE data and EST profiling indicated that it was expressed in human placenta and embryonic tissue. In addition, relative high expression of BCAR4 was observed in a subset of breast-cancer patients having lymph node metastasis at diagnosis and therefore a worse prognosis. In chapter 5 of this thesis further characterization of this gene is described.

Ectopic expression of BCAR4 resulted in proliferation of ZR-75-1 and MCF7 breast cancer cell lines in the presence of antiestrogens, indicating a causative role for this gene in the transformation of the cells from an antiestrogen-sensitive to an antiestrogen-resistant phenotype. ZR-75-1 cells, transfected to express BCAR4, produced tumors in nude mice in the absence of exogenous estrogen, while the parental cells were unable to do so. This establishes the transforming properties of BCAR4.

Quantitative RT-PCR on a large series of breast tumors may elucidate whether this gene also has predictive or prognostic values in breast cancer. In addition, generation of transgenic BCAR4 mouse models may shed some light on its role in tumor development and progression. To explore the potential of BCAR4 as a target in breast cancer therapy, it is necessary to further unravel its function and biological mechanism. Preliminary localization experiments with FLAG-tagged BCAR4 proteins showed their presence in the cytoplasm and to lesser extent on the cell membranes. Immunohistochemical analyses with suitable antibodies against BCAR4 and co-localization of the protein with different compartment and organelle-tracking dyes may assess localization of the wild-type protein. Determining the cellular localization of the protein may provide insight in its functional role.

Preliminary proliferation assays with small-molecule inhibitors indicated that MAPK14 (p38) may play a role in BCAR4 signaling. Independent validation of these assays may establish BCAR4 involvement in the well-known MAPK pathway. Further details about the biological mechanism of BCAR4 may be provided by knockdown experiments in various cell models, and identification of potential interaction partners of the protein using immunoprecipitation and mass spectrometry.

PATHWAY ANALYSIS

Ingenuity Pathway Analysis, KEGG, and literature studies were used to study the 7 genes causing tamoxifen resistance and 18 candidate genes recovered from our functional screen described in chapter 2. In addition, FGFR4 and TSC22D1, which were described to be associated with tamoxifen-therapy failure in breast cancer patients in chapters 3 and 4, were included. Figure 6.1 shows a simplified network of the genes described in this thesis, based on current knowledge. Three genes acetylserotonin methyltransferase-like (ASMTL), claudin 23 (CLDN23), and KIAA0513 were not eligible for network analysis. The

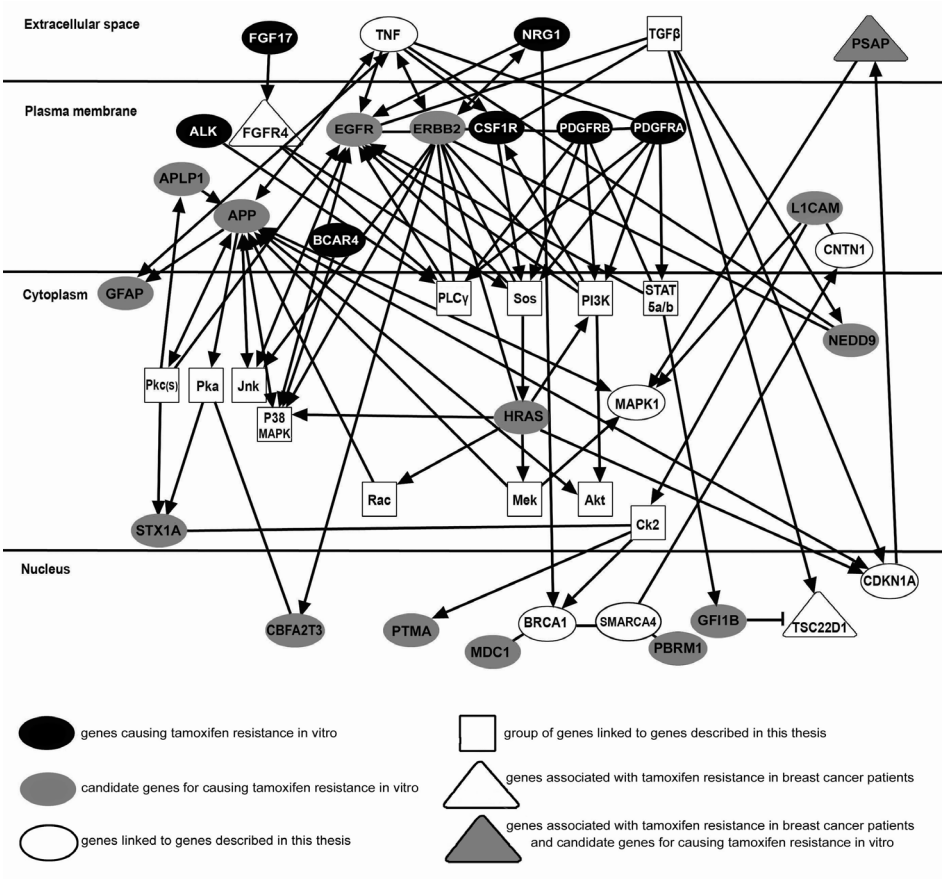

Figure 6.1

A simplified network of the genes described in this thesis and the genes linked to them. The results display a central role in tamoxifen resistance for genes involved in growth factor signaling and their downstream components in the MAPK and PI3K/AKT pathways.

results display a prominent role for growth factor signaling genes and their downstream targets in the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/AKT pathways. Indeed, these pathways have previously been reported in laboratory and clinical studies: elevated levels of NRG1, EGFR, ERBB2, and high levels of phosphorylated AKT, MAPK1 (ERK2), JNK, and MAPK14 (p38) were associated with tamoxifen resistance (10-21). In conclusion, pathway analysis shows that the genes identified in our functional screen for tamoxifen resistance can be placed in a network together with genes of pathways already known to play a prominent role in tamoxifen resistance.

FUTURE PERSPECTIVES

From the results in this thesis, it has become clear that functional profiling strategies may identify a variety of genes as causative for tamoxifen resistance. Because of their causative nature, these genes would provide potential targets for treatment of breast cancer patients resistant to tamoxifen therapy. However, not all of the genes will have an equally important function in tamoxifen resistance in patients as they do *in vitro*. For overcoming tamoxifen resistance in patients it is important to identify those genes that are causative and are associated with resistance in clinical specimens. Then the next step would be to develop and test drugs targeting these genes.

Identification of genes associated with resistance in clinical samples has been assessed by applying high-throughput profiling techniques, using for example cDNA microarrays (22). Comparison of the results of these kind of studies with the results of functional profiling strategies will provide the best candidate targets for therapy. The clinical relevance of two of such genes, TSC22D1 and PSAP, is described in chapter 4 of this thesis. The mRNA levels of TSC22D1 and PSAP were assessed in a set of primary breast tumors with clinical follow-up, and high levels were significantly associated with a shorter progression-free survival. Therapy outcome was even worse when patients had high mRNA levels of both genes. The clinical significance of these genes in breast cancer therapy makes them interesting candidates for future studies. Since their mechanisms of action have not been well characterized yet, further studies to elucidate the pathway through which these genes may cause tamoxifen resistance are recommended. To this end, *in vitro* models with ectopic expression of either one and both of these genes have to be generated. Experiments using microarray profiling will enable pathway analysis, possibly providing new drugable targets for therapy.

Another putative treatment target provided by our studies is FGFR4. FGFR4 was also demonstrated to be associated with resistance. Establishment of its causative role may be obtained by inducing ectopic expression of the gene in a tamoxifen-sensitive cell line or knocking out the gene in a FGF17-overexpressing tamoxifen-resistant cell line, and monitoring a conversion to tamoxifen insensitivity. Such establishment would make FGFR4 a novel potential target for patient-tailored therapy. Patients with breast tumors expressing high levels of FGFR4 and thus having a high chance of tamoxifen-therapy failure might then be treated with a small-molecule tyrosine-kinase inhibitor targeting FGFR4. FGFR inhibitor PD173074 has already been demonstrated to be able to reduce proliferation of the FGFR4-overexpressing tamoxifen-insensitive MDA-MB-453 breast cancer cell line (23). Further *in vitro* studies with PD173074 relating to tamoxifen therapy, and studies using animal models will be needed to establish its ability to overcome tamoxifen resistance. Results confirming this ability *in vitro* and *in vivo* would be a good basis for subsequent clinical trails.

It is known, however, that drug interference in important genetic pathways may cause serious side effects. Targeting a tumor-specific gene, like BCAR4 seems to be, would circumvent this problem. Therefore, further investigation of the biological mechanism of BCAR4 and its association with resistance in clinical samples, might provide us with a very promising new target for therapy.

CONCLUDING REMARKS

The results presented in this thesis contribute to the understanding of possible mechanisms causative for tamoxifen resistance in breast cancer. In addition, it was shown that these data may lead us to genes that are predictive for tamoxifen resistance in patients as well. Due to the broad spectrum of genes involved in resistance, it is clear that it will be very difficult to develop a new treatment strategy suitable for all tamoxifen-resistant patients. Instead, a combination of various treatment strategies targeting the most prominent escape pathways (including AIs, small molecule tyrosine kinase inhibitors, etc.) might be used for treatment. Furthermore, increasing the knowledge on mechanisms of resistance may contribute to the development of patient-tailored therapies based on the characteristics of the primary tumor in the future.

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CHAPTER 7

SUMMARY/SAMENVATTING

CURRICULUM VITAE

DANKWOORD

SUMMARY

Breast cancer is the most common cancer in women in the western world. Since estrogen-receptor positive breast tumors are often dependent on the female hormone estrogen, the antiestrogen tamoxifen is most extensively used in the treatment of breast cancer. Tamoxifen treatment inhibits tumor growth in half of the patients having estrogen-receptor positive tumors. However, the duration of this effect is limited and eventually the tumor will become resistant.

Chapter 1 generally describes the involvement of estrogens in breast cancer and the possible mechanisms of estrogen signaling in human cells. Although nowadays various therapeutic agents are available for endocrine therapy, like different SERMs, SERDs, AIs, and LHRH agonists, tamoxifen has been the mainstay for treatment of estrogen receptor positive breast cancers over the past thirty years. However, resistance to the drug, for which multiple mechanisms may be responsible, is an inevitable phenomenon. The aim of the research described in this thesis is to unravel the genes responsible for tamoxifen resistance in breast cancer.

Chapter 2 discusses a rapid functional approach applied for this purpose. Retroviral transduction of cDNA libraries in ZR-75-1 breast cancer cells, causing overexpression of random genes, revealed 73 genes that may be responsible for tamoxifen resistance. After application of stringent selection criteria 7 genes were considered to be responsible for tamoxifen resistance in breast cancer cells, and 18 genes were considered to be good candidate genes. Furthermore, the novel gene BCAR4 was identified. In addition, it has been shown that some of the genes identified in the functional screen had a predictive value for tamoxifen therapy in patients with recurrent breast cancer.

In **chapter 3** the mRNA levels of FGF17, and FGFR1-4 were assessed in 285 estrogen receptor-positive primary breast carcinomas using quantitative PCR. High levels of FGFR4 predicted a poor response on tamoxifen therapy and a shorter progression-free survival of the patients, independent from the traditional predictive factors.

Comparison of the list of genes revealed by the functional screen described in chapter 2 with an 81-gene signature derived from a microarray study for revealing genes predictive for tamoxifen response in breast cancer patients, identified two overlapping genes. In the study described in **chapter 4** mRNA levels of the two genes, TSC22D1 and PSAP, were measured in 223 tumors of patients with recurrent breast cancer using quantitative PCR. High mRNA levels of TSC22D1 and PSAP were significantly associated with shorter progression-free survival of the patients independent of the traditional predictive factors. Patients with tumors expressing high levels of both genes had the shortest progression-free survival. Identification of a genetic pathway in which both of these genes play a role, might elucidate possible novel targets for breast cancer therapy.

Chapter 5 describes further characterization of BCAR4, a novel gene causing tamoxifen resistance as identified in the functional screen described in chapter 2. It was shown that ectopic expression of BCAR4 caused resistance to antiestrogens in two breast cancer cell lines and tumor formation in nude mice. The gene is thought to be primate specific, and overexpression of the primate homologues also caused tamoxifen resistance of ZR-75-1 breast cancer cells. Fusion proteins of BCAR4 with FLAG-tags suggest protein localization in the cytoplasm and in the cell membrane. Experiments using small-molecule inhibitors have shown possible involvement of MAPK14 in the BCAR4 gene pathway. Our results suggest a possible role for this pathway in breast cancer progression.

Chapter 6 contains a general discussion about the results described in this thesis and future perspectives. The results revealed genes involved in tamoxifen resistance. Gene pathway analyses have demonstrated that all 7 genes responsible for tamoxifen resistance and 18 candidate genes were connected with genes involved in the MAPK and AKT pathways. The results suggest an important role for these genetic pathways in tamoxifen resistance in breast cancer.

Identifying genes involved in tamoxifen resistance and their corresponding genetic pathways is very important for improving diagnosis of breast cancer and enabling patient-tailored treatment in the future.

SAMENVATTING

Borstkanker is de meest voorkomende kanker bij vrouwen in de westerse wereld. Omdat oestrogeen receptor positieve borsttumoren voor hun groei vaak afhankelijk zijn van het vrouwelijk hormoon oestrogeen, wordt voor de behandeling veelal het anti-oestrogeen tamoxifen gebruikt. Bij de helft van de patiënten met dit soort tumoren blokkeert deze behandeling de groei van de tumor. Echter, de duur van dit effect kan sterk variëren en na verloop van tijd ontstaan resistente uitzaaiingen.

Hoofdstuk 1 is een algemene inleiding, waarin het belang van oestrogenen in borstkanker en de mogelijke mechanismen van het oestrogeen signaalpad besproken worden. Hoewel er tegenwoordig verschillende vormen van hormonale therapie beschikbaar zijn in de vorm van SERMs, SERDs, AIs en LHRH agonisten, is tamoxifen gedurende de afgelopen 30 jaar de voornaamste behandelingsvorm voor oestrogeen receptor positieve borstkanker geweest. Echter, resistentie tegen de therapie, waar verschillende mechanismen voor verantwoordelijk kunnen zijn, is onvermijdelijk. Het doel van het onderzoek, beschreven in dit proefschrift, is het identificeren van genen, die verantwoordelijk zijn voor tamoxifenresistente groei van borsttumoren.

Hoofdstuk 2 beschrijft een snelle aanpak om dit doel te bereiken. Door willekeurige genen uit bepaalde weefsels verhoogd tot expressie te brengen in ZR-75-1 borstkankercellen, zijn 73 genen geïdentificeerd die mogelijk verantwoordelijk zijn voor tamoxifen resistentie. Na het toepassen van strenge selectie criteria, heeft dit 7 genen en 18 kandidaat-genen opgeleverd die borstkankercellen tamoxifen resistent kunnen maken. Bovendien is van een aantal van deze genen aangetoond, dat indien de primaire tumor een hoge expressie had van de betreffende genen, patiënten minder goed reageerden op de behandeling met tamoxifen.

In **hoofdstuk 3** is de voorspellende waarde van FGF17 en de FGF receptoren 1-4 voor tamoxifentherapie beschreven. Door middel van kwantitatieve RT-PCR zijn in 285 oestrogeen receptor positieve primaire borsttumoren de mRNA waarden van deze genen gemeten. Hiermee is aangetoond, dat hogere expressie van FGFR4 geassocieerd is met een slechtere respons op tamoxifentherapie en een kortere progressievrije overleving van de patiënt, onafhankelijk van de traditionele voorspellende factoren.

De lijst van genen, die geïdentificeerd zijn in de experimenten beschreven in hoofdstuk 2, is vergeleken met een genprofiel van 81 genen met een voorspellende waarde voor tamoxifentherapie. Dit genprofiel is gemaakt met behulp van microarrays. Twee genen, TSC22D1 en PSAP, zijn via beide methoden geïdentificeerd als genen die gerelateerd zijn aan tamoxifen resistentie. In **hoofdstuk 4** is beschreven, dat in een serie van 223 oestrogeen receptor positieve primaire borsttumoren hoge expressie van deze genen geassocieerd is met een kortere progressievrije overleving van de patiënt, onafhankelijk van de traditionele voorspellende factoren. Hoge expressie van beide genen in dezelfde tumor voorspelde een nog kortere progressievrije overleving van de patiënt. Verder on-

derzoek naar het genetische pad waarin beide genen actief zijn, kan nieuw licht werpen op mogelijke nieuwe doelwitten voor de behandeling van borstkanker.

Verder bevond zich onder de 73 geïdentificeerde genen ook een nieuw gen, genaamd Breast Cancer Antiestrogen Resistance gene 4 (BCAR4), wat tot dan toe slechts voorspeld was met behulp van computer analyse. Experimenten beschreven in hoofdstuk 2 en in **hoofdstuk 5** hebben aangetoond dat BCAR4 inderdaad resistentie veroorzaakt tegen verschillende anti-oestrogenen in verschillende cellijnen en dat het tumorvorming in naakte muizen stimuleert. Het gen lijkt alleen in primaten voor te komen en transfectie van genetische homologen uit de rhesus aap en groene meerkat veroorzaakte eveneens tamoxifen resistentie in ZR-75-1 cellen. Fusie-eiwitten van BCAR4 en verschillende FLAG-tags suggereren dat het eiwit zich bevindt in het cytoplasma van de cel en bij het celmembraan. Experimenten met selectieve molecuulremmers duiden aan dat mitogen-activated protein kinase 14 (MAPK14/p38) mogelijk een rol speelt in het BCAR4 signaalpad. Onze resultaten ondersteunen een mogelijke rol voor dit pad in de progressie van borstkanker.

Hoofdstuk 6 omvat een algemene discussie over de resultaten uit het proefschrift en bespreekt mogelijkheden voor toekomstig onderzoek. De resultaten hebben genen onthuld die betrokken zijn bij tamoxifen resistentie. Door signaalpad analyses uit te voeren, is aangetoond dat de 7 genen verantwoordelijk voor tamoxifen resistentie en de 18 kandidaat-genen allemaal te verbinden zijn met genen uit de MAPK en AKT signaalpaden. Dit suggereert een belangrijke rol voor deze genetische paden in tamoxifen resistentie van borstkanker.

Het identificeren van de genen betrokken bij tamoxifen resistentie en de genetische paden waar zij invloed op uitoefenen is van groot belang voor de verbetering van de diagnose van borstkanker en voor het in de toekomst toe kunnen passen van een specifieke behandeling van borstkanker voor de individuele patiënt.

CURRICULUM VITAE

Daniëlle Meijer was born on the 7th of December 1979 in Rotterdam, The Netherlands. In 1998 she passed her secondary school exam at "Scholengemeenschap Johannes Calvijn" in Rotterdam, after which she started her study Medical Biology at the Free University in Amsterdam. During her first training period at the Department of Molecular and Cellular Neurobiology at the Free University in Amsterdam she studied gene expression changes in the prefrontal cortex of rats after morphine administration. She started her graduation research on identifying genes responsible for tamoxifen resistance in breast cancer at the Department of Pathology at the ErasmusMC in Rotterdam in 2002. In 2003 she received her master's degree in Medical Biology after which she continued working on the topic of her graduation research as a PhD student at the Department of Pathology under the supervision of dr. ir. L.C.J. Dorssers and prof. dr. J.W. Oosterhuis. In 2005 she received the 'AACR-Pezcoller Foundation Scholar-in-Training Award' for her 'Functional screen for identifying genes responsible for tamoxifen resistance resistance in human breast cancer cells' described in chapter 2 of this thesis. This paper was orally presented at the 2005 Annual Meeting of the AACR in Anaheim (CA, USA). Her publication stating that 'FGFR4 predicts failure on tamoxifen therapy in women with recurrent breast cancer' described in chapter 3 was orally presented at the Annual Meeting of the AACR in 2006 in Washington (DC, USA). As from November 2007 Daniëlle is working as a post-doctoral researcher at the Department of Pathology at the Leiden University Medical Center studying the potential of the estrogen-signaling pathway as a target for chondrosarcoma treatment.

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