BCAR1 and Anti-estrogen Resistance of Human Breast Cancer

BCAR1 en anti-oestrogeen resistentie van humane borstkanker

Proefschrift

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What we anticipate seldom arrives; what we least expect generally happens.

Benjamin Disraeli

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Abbreviations

aa	amino acids
BCAR	breast cancer anti-estrogen resistance
BSA	bovine serum albumin
cDNA	complementary DNA
EGF(R)	epidermal growth factor (receptor)
ER(α, β)	estrogen receptor (α,β)
ERE	estrogen response element
FAK	focal adhesion kinase
FGF	fibroblast growth factor
IGF	insulin-like growth factor
ITT	*in vitro* transcription translation
kb	kilobase
kDa	kilodalton
LOH	loss of heterozygosity
LTR	long terminal repeat
MMP	matrix metalloproteinase
mRNA	messenger RNA
ORF	open reading frame
p130Cas	p130 Crk-associated substrate
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PgR	progesterone receptor
PAGE	polyacrylamide-gel electrophoresis
RIM	retroviral insertional mutagenesis
RT-PCR	reverse transcriptase PCR
SH(2,3)	Src homology 2 or 3 domain
TGF(α, β)	transforming growth factor(α, β)
TNM	tumor-node-metastasis
UTR	untranslated region
VEGF	vascular endothelial growth factor

CHAPTER 1

Introduction

1.1 Aim of this study

One of the most widely used and effective agents in the treatment of metastatic breast cancer is the anti-estrogen tamoxifen. The efficacy of therapy with this low toxic anti-hormonal drug is based on the dependence of many breast tumors on estrogen for growth and survival. The mechanism of action of anti-estrogens relies on their ability to compete with estrogens for binding to the estrogen receptor (ER) and to block cell proliferation in hormone-dependent cells (*1,2*). Many breast carcinomas express the estrogen receptor α (ERα) and are eligible for treatment with tamoxifen. Nearly 50% of ER-positive tumors respond to tamoxifen treatment by tumor regression or long-term disease stabilization. However, the response duration is limited due to the inevitable development of tamoxifen-resistant tumors (acquired resistance). Moreover, half of the patients with ER-positive tumors do not respond to tamoxifen treatment (intrinsic resistance). Tamoxifen resistant proliferation of human breast cancer cells results from yet unresolved mechanisms. Presumably, there are several mechanisms capable of bypassing estrogen dependency and causing tamoxifen resistance. The working hypothesis for this study was that (epi)genetic alterations occurring in the tumor cells might play a role in intrinsic and acquired resistance to tamoxifen treatment.

In search of genes involved in tamoxifen resistance of breast cancer cells, an *in vitro* model system has been developed which mimics the clinical situation. Accordingly, the breast cancer anti-estrogen resistance 1 (*BCAR1*) locus has been identified, harboring a gene involved in tamoxifen resistance *in vitro*. The objective of this study was to identify and characterize the gene in the *BCAR1* locus, and to delineate the importance of BCAR1 in clinical tamoxifen resistance.

1.2 Breast cancer

Breast cancer is the most frequently diagnosed cancer among women in the Western world and affects women of all ages. The ultimate goals in studying breast cancer biology are the prevention of breast cancer and the reduction of

mortality by identifying women at risk for the disease, predicting the prognosis of existing disease, predicting response to different therapies, and optimizing the treatment of breast cancer.

1.2.1 Concepts of breast tumorigenesis

Development and progression of breast cancer

The breast is the most common site for development of cancer in the female. The breast structure, the breast volume and the extreme sensitivity to endocrine influences, all predispose this organ to the development of breast cancer. The development of a breast tumor usually takes quite a long time. It is thought that 30 doublings in size are required for a tumor to grow from a single cell, 10 microns in diameter, to a multicellular mass of 1 cm in diameter (*3*). The majority of the primary breast carcinomas are considered to have their origin in the epithelial cells of the terminal ductal lobular unit presenting as either intraductal or intralobular lesions. Breast cancer is thought to arise via a multistep process. The sequential nature of this process has been suggested by the observation that invasive breast carcinomas are often preceded by a series of intermediate hyperplastic (with and without atypia) and neoplastic (*in situ* carcinoma) stages, frequently followed by metastatic cancer (*4*). However, studies on the malignancy process in the breast have been complicated by the heterogeneity of the preinvasive lesions.

Relatively little is known about the control mechanisms of cellular proliferation in normal breast epithelium. Estrogens and progestins are ovarian steroid hormones that are essential for the growth and differentiation of normal breast tissue, and are involved in the development and progression of breast cancer (*5,6*).

Many invasive breast carcinomas give rise to distant metastases, particularly in bones, soft tissues, and visceral organs such as lung and liver, and in the central nervous system especially the brain. The metastatic capacity of breast tumors

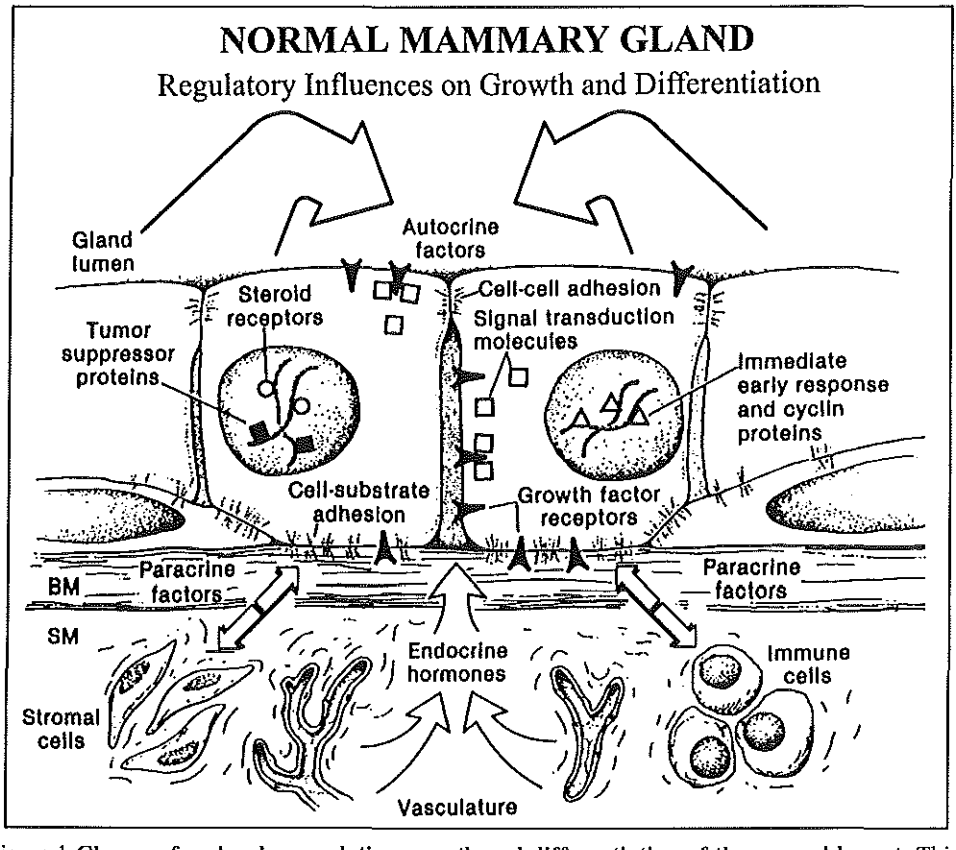

Figure 1 **Classes of molecules regulating growth and differentiation of the normal breast.** This figure represents a generalized scheme of the range of regulatory influences in the mammary gland signal transduction from receptors, cell-cell, and cell-substrate adhesion molecules that become perturbed in cancer. Immediate early response genes (nuclear protooncogenes), cyclin-CDK-CDKI complexes, and tumor suppressor genes may also be disregulated and/or mutated. In addition, disregulated production of growth factors may lead to abnormal stromal, vascular, and immune function, contributing to disease progression and metastasis. *BM*, basement membrane; *SM*, stromal matrix (reproduced from R.B. Dickson (*7*))

requires the following interdependent processes (*8*):

- detachment of cancer cells from their original location (loss of cellular adhesion)
- cancer cell migration

- invasion of cancer cells into the surrounding tissue, requiring adhesion to and subsequent degradation of extracellular matrix (ECM) components
- access of cancer cells to blood and lymphatic vessels (intravasation)
- surviving the immune system
- adhesion to and invasion through the endothelium, allowing colonization at distant sites in the body.

During cancer invasion and metastasis with secondary tumor growth, tumor cells cross the basement membranes and the surrounding extracellular matrix. An early step in this invasive process is the adhesion of the tumor cells via cell-surface receptors (e.g. integrins, uPAR) to proteins such as laminin, type IV collagen, vitronectin, and fibronectin in the basement membrane. Subsequent to adhesion, traverse of the biological barriers by the tumor cells is accomplished by a cascade of extracellular, tumor-associated, proteolytic enzyme activities that locally degrade the components of the basement membranes and extracellular matrix (*9*). Several tumor-associated proteases like plasminogen activators, matrix metalloproteinases, and cathepsins are thought to play a role in these degradation processes (*8*).

Molecular genetics of breast cancer

During the process of tumorigenesis of both sporadic and familial breast cancer, mammary cells undergo various genotypic and phenotypic changes that allow these cells to bypass the normal controls of tissue homeostasis. The accumulation of 5 to 10 genetic and epigenetic alterations is thought to promote malignancy. Progression of the tumor to an aggressive, metastatic cancer depends on additional (epi)genetic changes that permit invasion, migration, and angiogenesis, and escape of the immune system.

Somatic genetic abnormalities that have been detected in breast tumors are among others the amplification of oncogenes (*MYC, ERB-B2, cyclin D1* or *CCND1*, and *AIB1*), the mutation of the tumor suppressor genes *TP53* and *E-cadherin* (*CDH1*), and loss of heterozygosity (LOH) at chromosomes 1, 3p, 6q, 7q, 8p, 9p, 10q, 11,

13q, 16q, 17, 18q, 22q and X (*10*). LOH may entail complete loss or inactivation of tumor suppressor genes.

Apart from genetic alterations, gene expression in cells may become altered through non-mutational processes that influence the expression of genetic information. Such epigenetic events are changes in the regulation of gene transcription that occur without a change in the primary DNA sequence. Rather there is a modification of the promoter region of a gene by (de)methylation, or chromatin-mediated and long-range interactions between distant chromosomal elements.

Gene mutations have been identified as the cause of autosomal dominant inherited predisposition to breast cancer (and ovarian cancer). The recent discovery of the tumor suppressor genes *BRCA1* and *BRCA2* have significantly advanced the molecular understanding of hereditary breast cancer. Mutations in the *BRCA1* and *BRCA2* genes are rare at the somatic level, but inheritance of a germline mutation in one of these genes predisposes to breast cancer (*11-14*). Breast tumors of patients who have inherited a germline mutation in the *BRCA1* or *BRCA2* gene show a larger number of additional genetic abnormalities compared with sporadic tumors. This observation may be explained by the discovery that the protein products of these hereditary breast cancer genes associate with the DNA repair machinery of the cell (*15,16*). Whether these additional alterations are involved in the development and tumor progression in carriers of mutations in the *BRCA1* and *BRCA2* genes is unknown.

Incidence and epidemiology

Approximately 10% of all women in western countries will, during their lifetime, develop breast cancer (*17,18*). Therefore, breast cancer is the most frequently occurring malignancy in females. Breast cancer also may affect males, but the incidence is about 1% of that in females (*3*). Breast cancer is rare under the age of 20, but thereafter the incidence rises steadily throughout life. In addition to age differences, there are striking geographic differences in the incidence of the

disease. In The Netherlands, which is among the countries with the highest incidence, each year approximately 10 000 new cases of breast cancer are diagnosed, and about 3500 women die as a result of this disease. A 5% yearly increase in incidence of breast cancer in women of 45 years and older has been observed between 1989 and 1994. This is most probably due to the increasing life-expectancy of women, change in reproductive and endocrine risk factors like higher age at birth of first child, decrease in the number of children, decrease in the average number of nursing months, and younger age at menarche (longer duration of ovarian activity). Moreover, part of the increase in incidence is caused by the introduction of the national breast cancer-screening program, which has led to earlier diagnosis of tumors in the breast. Since 1988, the mortality as a result of breast cancer has remained constant despite the detection of an increasing number of individuals with breast cancer. In conclusion, the survival rate has improved both by earlier detection and treatment, which has become more effective since the introduction of additional chemo- and hormonal therapy at different stages of the disease. Still, breast carcinoma is the cause of death in approximately 22% of women dying of some sort of cancer (*19*).

Epidemiological studies have clearly established the role of family history as an important risk factor for breast cancer. Next to gender and age, a positive family history is the strongest known predictive risk factor for breast cancer. Members of families showing a high incidence of cancer, due to specific mutations in single cancer susceptibility genes (*BRCA1* and *BRCA2*), which are mutation carriers have a 60-85% risk of developing breast cancer. These hereditary breast cancers comprise 5-10% of all breast cancer cases.

Pathology and clinical staging

A great variety of tumors may occur in the female breast that is made up of mesenchymal connective tissue, epithelial structures and fat. Neoplasms may arise from stratified squamous epithelium, glandular structures and mesenchymal connective tissue. Also combinations of different tumor types occur. Breast tumors are histologically classified according to criteria of the World Health

Organization (*20*) either as benign or as malignant. However, carcinomas (malignant tumors of epithelial origin) predominate in importance due to their aggressive behavior. A histopathologic distinction is made between carcinomas that have not penetrated the limiting basement membrane (non-invasive carcinomas) and those that have (invasive carcinomas). An overview of a selected range in breast tumor types is provided by the following classification:

Table 1. Histological classification of breast tumors
(adapted from (*20*))

Malignant epithelial tumors	
Type	Subtype
Non-invasive	Intraductal carcinoma *in situ*
	Lobular carcinoma *in situ*
Invasive	Ductal carcinoma NOS[a]
	Lobular carcinoma
	Mucinous carcinoma
	Medullary carcinoma
	Papillary carcinoma
	Tubular carcinoma
	Adenoid carcinoma
	Secretory (juvenile) carcinoma
	Apocrine carcinoma
	Carcinoma with metaplasia
Paget's disease of the nipple	

Mixed connective tissue and epithelial tumors
Type
Fibroadenoma
Phyllodes tumor (Cystosarcoma phyllodes)
Carcinosarcoma

[a]NOS: not otherwise specified

Most tumors arise in the ductal epithelium (70%), whereas the lobules (8%) are rare sites of origin. Intraductal carcinoma is characterized by four growth patterns: solid, comedo (ropey cords that are palpable and from which necrotic tissue can be extruded with slight pressure when the ducts are transected), papillary (central connective tissue framework covered by one or two layers of small, regular, cuboidal epithelial cells), and cribriform (ducts that are more or less filled with masses of anaplastic cells, creating small glandular spaces). Mixtures of these four patterns usually occur, although any one may predominate in a given tumor.

Invasive ductal carcinoma NOS is the most common tumor type of the breast. Microscopic appearances vary widely. The tumor cells are round to polygonal, compressed, and contain fairly uniform, small, dark nuclei with few mitotic figures. Tumor cells are arranged as nests, cords, and gland-like structures. The tumor can be multicentric and foci of intraductal carcinoma may be present. At the margins of the tumor the neoplastic cells can be found to infiltrate the surrounding tissue.

The lobular breast carcinomas arise in the terminal tubular glands of the lobules. The cells are usually relatively uniform and of small or moderate size, with faintly staining cytoplasm and finely structured, rounded nuclei exhibiting no or very few mitoses. Lobular carcinoma *in situ* is a dangerous lesion because of its frequent multicentricity, and association with invasive ductal and lobular carcinomas. Lobular carcinoma involves both breasts more frequently than other histologic types. In invasive lobular carcinoma the cells grow in a single file, forming a linear arrangement, or appear individually embedded in fibrous tissue. The other forms of invasive epithelial tumors, as listed above, occur rarely.

The most common solid benign tumor of the female breast is the fibroadenoma that grows as mammary dysplasia composed of both connective fibrous and glandular tissue. Fibroadenomas usually appear in young women as a discrete, usually solitary, freely movable nodule, 1 to 10 cm in diameter. Infrequently, fibroadenomas may grow to massive proportions, reaching diameters of 10-15

cm, so-called giant fibroadenomas. They almost never become malignant, but there is an increased risk in women with a familial occurrence of breast cancer. Histologically, there is a loose fibroblastic stroma containing duct-like, epithelium-lined spaces of various forms and sizes. Although in some lesions the ductal spaces are open, round to oval, and fairly regular, others are compressed by extensive proliferation of the stroma.

The phyllodes tumor (cytosarcoma phyllodes) is much less common than the fibroadenoma and is thought to arise from intralobular stroma, and not from preexisting fibroadenomas. This neoplasm has a foliated structure and is composed of connective tissue and epithelial components, characterized by a greater connective tissue cellularity than the fibroadenoma. These tumors are usually large. On histologic appearance it is difficult to predict the biologic behavior of phyllodes tumors. Based on a number of criteria (frequency of mitoses, infiltrative margins, cellular atypia, and cellularity), three separate categories (benign, borderline, and malignant) are defined.

Table 2. General characteristics of the clinical stages

Stage 0	*In situ* cancer (*in situ* lobular, pure intraductal, and Paget's disease of the nipple without palpable tumor)
Stage I	Tumor 2 cm or less in greatest diameter and without evidence of regional or distant spread
Stage II	Tumor more than 2 cm but no more than 5 cm in greatest diameter, with regional lymph node involvement but without distant spread
Stage III	Tumor of more than 5 cm in diameter, infiltration, ulceration or thickening of the skin, or pectoral muscle or chest wall attachment, usually regional lymph node involvement, distant metastasis may or may not be suspected
Stage IV	Tumor of any size with or without regional spread but with evidence of distant metastases

As a guide for therapy selection, patients with breast cancer are grouped into one of the stages according to criteria defined by the Union International Contre le Cancer (UICC). This classification is based on three clinical patient characteristics: diameter of the tumor (T), involvement of lymph nodes (N), and the presence of distant metastasis (M), hence the term TNM-classification.

1.2.2 Steroid hormones, growth factors and receptors

The development of the breast, the tumorigenesis of the breast epithelium, and the progression of breast cancer are regulated by hormonal factors. Examples of these factors are the steroid hormones, peptides, and other molecules produced by the secretory cells of the ovaries, pituitary gland, endocrine pancreas, thyroid gland, and adrenal cortex. After their initial binding to either nuclear or cell-surface receptors, the hormonal factors regulate cellular function.

Estrogen receptor

Estrogens play an essential role in the development, progression, treatment and outcome of estrogen receptor-positive mammary carcinomas (*21-23*). Estrogens exert their function by interacting with the estrogen receptor (ER) protein, resulting in the transcriptional activation of specific sets of genes. Transcriptional activation of genes by estrogens is mediated by the interaction of the activated ER with specific DNA sequences (estrogen response elements, EREs) located in the promoter of these genes, and with other components of the transcription machinery. Two ER genes have been identified that both belong to the superfamily of nuclear hormone receptors. The first, ERα and generally referred to as ER, was cloned from a human breast cancer cell line and is also expressed in the normal mammary gland (*24*). Subsequently a highly homologous gene, ERβ, was cloned from prostate tissue (*25*). Estrogens are known to bind ERβ with affinity similar to ERα (*26*), and the transcriptional activation via the ERE is identical for both receptors (*25,27,28*). There is evidence that ERβ is expressed in normal and malignant breast cells (*29,30*). Recently, ERβ mRNA was shown to be significantly up-regulated in patients with tamoxifen resistant breast cancer

and tamoxifen resistant breast cancer cell lines, suggesting a role for ERβ as a poor prognostic factor in breast cancer (*31*). ERα is a key transcription factor for genes such as the progesterone receptor (PgR) and pS2 (a trefoil peptide). Expression of PgR and pS2 in ERα-positive breast cancer is evidence of an intact estrogen response pathway and an indicator of hormone responsiveness (*32,33*). About two-third of primary breast tumors are ERα-positive. The expression of ERα has important implications for the biology of breast carcinomas. Tumors expressing the ERα at a detectable level tend to grow more slowly and are more highly differentiated compared to tumors with no detectable ERα expression. ERα-positive tumors are more likely to occur in post-menopausal women, whereas ERα-negative breast cancers occur more frequently in young women. Estrogens promote the growth and survival of normal breast tissue as well as ERα-positive carcinoma cells. Nevertheless, ERα-negative tumors have in general a worse clinical outcome compared to ERα-positive tumors. It has been hypothesized that ERα-negative tumors behave more aggressively and poorly differentiated, because they have acquired the ability to bypass the ERα pathway for growth and survival. Several groups have reported that ERα loss appears to occur in some tumors over a period of time. Approximately 20% to 25% of tumors originally determined to be ER-positive, were found to be ERα-negative at the time of recurrence (*34*). Some patients who had been classified as ERα-negative based on the primary tumor had ERα-positive recurrences (*35*). These findings suggest that clonal expansion *in vivo* may result not only in a loss of ERα, but also in an apparent gain of ERα expression (*35*). For simplicity, ERα will further on be referred to as ER.

Progesterone receptor

Two forms of the PgR (PgR-A and PgR-B) mediate the biological response to progesterone. These two isoforms are transcribed from distinct, estrogen-inducible promoters within a single-copy progesterone receptor gene, and both function as a transcription factor. Several studies suggest that the PgR receptor isoforms may function differently and that the relative levels of expression of

PgR-A and PgR-B could be a determinant of hormone sensitivity (*36,37*). PgR-positive tumors are considered to express both the A and B isoform, albeit at different ratios. About 50% of all ER-positive breast tumors are also positive for PgR. These double-positive tumors exhibit the highest response rate to endocrine therapy (about 75%), whereas less than one third of ER-positive/PgR-negative tumors initially respond (*38,39*). This finding may simply reflect the fact that ER is a key transcription factor for the expression of PgR. Thus, lack of PgR expression in ER-positive tumors may be indicative of a nonfunctional or aberrantly functioning ER that is not likely to be affected by anti-estrogens. It is not surprising that nearly all ER-negative tumors also lack PgR and rarely respond to tamoxifen.

Unlike ER status, the level of PgR expression is more related to menopausal status of patients than to patient age. Premenopausal patients have a higher PgR level, which is probably induced by the higher estrogen concentration (*40*). Like ER, well-differentiated tumors are more likely to be PgR-positive than poorly differentiated tumors. Tumors that are initially PgR-positive frequently convert to PgR-negative over time. This change in receptor status is accompanied by more aggressive tumor behavior, loss of endocrine control, and poor survival (*41*).

Growth factors and peptide hormones

A variety of mouse experiments have demonstrated the ability of epidermal growth factor (EGF) to promote tumorigenesis, and several members of this family (both ligands and receptors) are commonly overexpressed in human breast cancer (*42*). The EGF family includes four transmembrane tyrosine kinase receptors, HER-1 or EGFR, HER-2, -3, and -4), and several growth factors including EGF, TGF-α, amphiregulin, and cripto-1. Twenty to fifty percent of breast cancers overexpress the receptors HER2 or EGFR. High expression of EGFR is often accompanied by a low level or absent expression of ER, suggesting a mechanistic relation between up-regulation of EGFR and hormone-independence (*43-45*).

Several other families of growth factors (such as TGF-βs, IGFs, PDGFs and FGFs) and their binding receptors have been under study for several years, and were shown to be related to breast cancer (*7,46-48*).

Other hormonal systems and growth factors, like somatostatin, inhibins, activins, androgens, vitamin D and thyroid hormones could also affect breast cancer initiation and progression. However, their exact contribution to normal and/or malignant breast cell growth is as yet poorly characterized.

1.2.3 Treatment of breast cancer

The treatment of breast cancer, depending on the stage of disease progression, consists of surgery, radiotherapy, chemotherapy, endocrine therapy and combinations thereof. Most treatments are often accompanied by the problem of side effects and the development of therapy resistance.

The current procedure for operable breast cancer (stages I and II) is the modified radical mastectomy, consisting of a total mastectomy with complete axillary dissection, or breast conserving therapy (lumpectomy and radiotherapy) for relatively small tumors. Postoperative radiotherapy is essential as a complement to limited surgery for tumors of small size or when pathological examination shows that the disease is so extensive that local recurrence is likely.

For patients with early-stage disease without evidence of distant metastases, additional (adjuvant) systemic treatment following mastectomy is often recommended with the goal to eradicate micrometastases which may be present at the time of diagnosis and to cure patients (*49*). Clinical research has clearly demonstrated that adjuvant therapy improves disease-free and overall survival of early-stage breast cancer patients (*50,51*). To improve the clinical outcome of patients with advanced disease, several systemic therapeutic strategies in the form of chemotherapy and/or endocrine therapy have been instituted. Once breast cancer has metastasized, the patient can no longer be cured. The goals of systemic treatment of patients with metastatic breast cancer are to prolong survival and palliate symptoms related to the disease.

Chemotherapy

As chemotherapy, combinations of cytotoxic agents are being used including CMF (Cyclophosphamide, Methotrexate, 5-Fluoroacil), FAC (5-Fluoroacil, Adriamycin, Cyclophosphamide) or AC (Adriamycin, Cyclophosphamide). However, the efficacy of chemotherapy is often limited due to resistance, even when high dosages have been used. Usually, patients non-responsive to chemotherapy exhibit resistance to multiple cytotoxic agents of different structure. This multiple drug resistance (MDR) may be caused by various mechanisms (*52*). Intracellular mechanisms that can reduce the drug concentration at the molecular level include membrane-bound drug efflux pumps such as MDR1/p-glycoprotein or the multidrug resistance-associated protein MRP, clearing of agents in cytoplasmic vesicles, and detoxification. A novel transport mechanism leading to resistance to several anticancer drugs is suggested by the recently isolated breast cancer resistance protein (BCRP) (*53*). BCRP is an adenosine triphosphate-binding cassette transporter and overexpression of BCRP in drug-sensitive breast cancer cells conferred resistance to mitoxantrone and anthracyclines. Several other pharmacokinetic mechanisms responsible for the drug resistance can reduce the amount of active drug arriving at the cancer cells. These include metabolic inactivation and poor penetration of drugs through the interstitial tumor tissue.

Endocrine therapy

Some forty years ago, Lerner and coworkers (1958) discovered the first nonsteroidal anti-estrogen, and Jensen and Jacobsen (1960) identified a target for drug action, the ER as reviewed by MacGregor (*54*). Endocrine therapy with the anti-estrogen tamoxifen is now considered the first-line endocrine treatment option for the majority of patients with advanced breast cancer and is an integral component of adjuvant therapy for many patients with early-stage breast cancer. Tamoxifen was approved by the Food and Drug Administration in 1977 for the treatment of women with advanced breast cancer and several years later for adjuvant treatment of primary breast cancer. Tamoxifen exerts its antitumor effects by competitive inhibition of estrogen binding to the intracellular estrogen

receptor (*55*). As a consequence, tamoxifen inhibits the expression of estrogen-regulated genes. The net result is a block in the G1 phase of the cell cycle and a slowing of cell proliferation. Tumors may then regress because of this altered balance between cell proliferation and ongoing cell death. However, tamoxifen has also been shown to exhibit weak agonistic activity. The dual effects of tamoxifen in the same tissue have not been explained but could be caused by estrogen receptor-interacting proteins that may be specific for certain genes and cell types. Tamoxifen is a well-tolerated drug with minor side effects, but brings about an increased risk of endometrial cancer (*51,55,56*).

Adjuvant therapy with tamoxifen is used either alone or in combination with chemotherapy for early-stage ER/PgR-positive breast cancer in both pre- and postmenopausal women and has been shown to reduce recurrence rates, mortality, and the incidence of contralateral breast cancer (*51,57*). Evidence exists that tamoxifen treatment with a duration of 5 years yields an improved disease-free survival as compared to a 2-year duration (*51*).

Treatment with tamoxifen is indicated for postmenopausal women with metastatic breast cancer, for premenopausal women with ER-positive metastatic breast cancer, and for men with metastatic breast cancer. In postmenopausal women with advanced breast cancer, tamoxifen induces objective responses in about one-third of unselected patients; a higher response rate is observed in women with ER-positive tumors (*58*). In the absence of ER and PgR, response to tamoxifen is observed in only approximately 10% of breast cancer patients, while the presence of substantial amounts of both ER and PgR predict that a response to tamoxifen will occur in up to ca. 75 % of such patients (*39*).

Tamoxifen may also be beneficial in preventing the development of breast cancer in women at high risk of the disease (*59*). Tamoxifen appears most beneficial for younger women with an elevated risk of breast cancer (*60*). Currently, several clinical trials are being performed to establish the benefit of anti-estrogens in preventing breast cancer.

Therapeutic response to endocrine therapy of patients with advanced disease is assessed according to the International Union Against Cancer (UICC) criteria (*61*). Any reported objective response, i.e. complete remission or partial remission, has to be confirmed at two different visits. Stable disease should be of a minimum duration of 6 months from start of treatment to be considered as a response. Patients whose tumor progresses within 3 months of starting tamoxifen therapy are classified as having progressive disease. Overall, approximately 50% of patients with metastatic disease respond to tamoxifen therapy. There is also a substantial fraction of patients whose disease becomes stable, neither progressing nor regressing, for a clinically significant period of time. Patients in whom the cancer remains static for at least 6 months have a statistically similar survival as patients whose tumors show a partial response (*33,62,63*). Patients whose tumors show objective response to tamoxifen treatment are defined as responding to the tamoxifen treatment and those that do not are referred to as having intrinsic resistance. Significantly, patients who are intrinsically resistant to a first-line tamoxifen therapy have a poor chance (about 20%) of responding to second-line agents. In contrast, patients with acquired resistance to first-line tamoxifen treatment have a 50% chance of responding to further endocrine manipulation. Other endocrine therapies for postmenopausal women include pure steroidal anti-estrogens (ICI 182,780), which exhibit virtually no estrogen-like effects, high dose progestins, antiprogestins, androgens, and aromatase inhibitors that act through different mechanisms. Because breast cancer is a progressive disease and the development of drug resistance is common, these alternative endocrine therapies are given sequentially as second-line and/or third-line treatment.

1.2.4 Prognostic and predictive factors

In breast cancer, prognosis is influenced by several variables called prognostic factors. Prognostic factors are clinical and pathological features as well as (molecular) biological characteristics used to predict the clinical course (disease-free survival, post-relapse survival, and overall survival of patients) of breast cancer at the time of primary treatment. The ability to predict the biological

behavior of breast tumors allows the selection of optimal treatment and follow-up strategies. Systemic therapy is offered to patients with metastatic disease and patients with primary breast cancer and a poor prognosis. For the choice of systemic treatment (endocrine therapy or chemotherapy), predictive factors are useful in predicting tumor sensitivity or resistance to these therapies.
All known predictive factors also appear to be prognostic, but not always *vice versa* (*64*).

Traditional prognostic factors

Age and menopausal status

Patients who develop breast cancer at a young age are considered to be at high risk of relapse (*65*). For postmenopausal patients age was not associated with the rate of relapse, and older premenopausal women had the best prognosis (*66*).

Tumor size and nodal status

The most important routine prognostic factor is the TNM stage of the tumor. The size of the primary tumor is positively correlated to the risk of recurrence and becomes even more powerful with regard to predictiveness of survival when combined with the nodal status. Next to the presence of tumor cells in the lymph nodes, the number of tumor-involved lymph nodes is of prognostic importance (*67*). Patients with few involved nodes have a better prognosis than those with many (≥3).

The histological grade of the tumor

The most frequently used histological breast tumor grading systems is that of Bloom and Richardson (*68*), which is based on tubule formation, nuclear grade, and mitosis. In several studies involving node-negative breast cancer patients, a poor tumor grade was found to be associated with a short disease-free period as reviewed by Foekens (*65*). This indicates a more aggressive behavior of these tumors.

Estrogen and progesterone receptor

Both ER and PgR are of prognostic value for short-term but not for long-term outcome of the patient (*69*). The main clinical utility of determining ER-status is to identify patients with ER-negative tumors and spare them ineffective treatment. The steroid receptors have strong predictive value for endocrine therapy: high levels of ER and PgR in the primary tumor predict a relatively good response to endocrine therapy, especially for postmenopausal women.

Modern prognostic factors

Proliferation indicators and DNA content

Tumor proliferative activity, expressed as flow-cytometric S-phase analysis, thymidine labeling index (TLI), or mitotic index (Ki-67/MBI-1), has become a widely accepted determinant of rapid relapse. Highly proliferative tumors are generally associated with shorter disease-free interval and overall survival. DNA-ploidy of breast tumors is measured by flow cytometry. Aneuploidy together with a high number of cells in the S-phase fraction was found to be associated with poorly differentiated carcinomas, larger tumor-diameter, ER-negative tumors, and lower survival rates (*70*). Aneuploidy combined with high proliferation indices indicate a greater chance of poor response to endocrine therapy in metastatic breast cancer (*71*).

EGFR and c-ErbB2

EGFR status has been shown to be a marker of poor prognosis in patients with primary breast cancer, especially in patients with node-negative breast cancer (*72*), but the prognostic power decreases with longer follow-up (*73*). Of great clinical significance is the associaton of EGFR with endocrine resistance (*74*). The c-ErbB2 oncogene codes for a growth factor receptor (the HER2/*neu* protein) and is a member of a family of EGFR related proteins. Although this receptor is expressed to a very limited degree in normal cells, it is overexpressed in 20-35% of breast tumors (*64*). Overexpression of the HER2/*neu* protein resulting from amplification of the c-ErbB2 oncogene predicts a poorer prognosis both in terms of disease-free interval and overall survival in patients with positive axillary

nodes (*75*). There is growing evidence that c-ErbB-2 expression is an important predictor of resistance to tamoxifen in primary (*76*) and metastatic breast cancer (*77,78*).

Tumor suppressor gene p53

The p53 gene and its protein product appeared to be involved in cell cycle control, DNA repair, apoptosis, cellular differentiation, senescence, and angiogenesis. In general, p53 mutations or overexpression are associated with a poor treatment outcome, also in node-negative disease (*73,79*). For patients with recurrent breast cancer, the presence of a p53 gene or protein alteration predicts poor response to tamoxifen therapy (*80,81*).

Invasion markers

Important enzymes involved in invasion and metastasis are cathepsins B, D and L, and urokinase-plasminogen-activators (uPA). High tumor levels of cathepsin D are related with an increased rate of relapse in patients with primary breast cancer (*82*). Cathepsin B and L also play an important role and have prognostic value (*83*). uPA seems to be one of the strongest prognostic factors in breast cancer (*84*). The effect of uPA is, at least partly, modulated by the presence of specific inhibitors, plasminogen activator inhibitor type I (PAI-1) and type 2 (PAI-2). PAI-1 also appears to be a strong prognostic factor in breast cancer (*85*), whereas the importance of PAI-2 has only been indicated in a few studies (*86*).

Tumor angiogenesis

Angiogenesis, the induction of neovascularization, is necessary for tumor growth and metastasis. Angiogenic activity of a tumor may result from downregulation of inhibitors of angiogenesis or up-regulation of endothelial growth factors. The majority of studies show that angiogenesis is an important new prognostic factor in early-stage breast carcinoma. A worse prognosis was shown in several studies for patients with high angiogenic activity (*87-89*). The predictive value of markers for angiogenesis has yet to be established.

1.2.5 Tamoxifen resistance

Tamoxifen therapy of breast cancer has been established as a valuable therapeutic strategy. Over half of patients with ER-positive disease show no response to tamoxifen (intrinsic resistance); others develop acquired resistance to tamoxifen. The majority of progressing tumors of patients on tamoxifen treatment continue to express ER, indicating that mechanisms for resistance other than ER loss are common in breast cancer (*90*). An understanding of the mechanisms by which this resistance occurs should provide alternative approaches to improve therapy effectiveness. Many possible mechanisms of tamoxifen resistance have been proposed and have been or are being evaluated, as considered below.

Pharmacological changes

Tamoxifen undergoes metabolic conversion to the two main metabolites, 4-hydroxytamoxifen and N-desmethyltamoxifen. It has been suggested that tumoral uptake and retention of tamoxifen metabolites that are either less potent anti-estrogens or exhibit estrogenic properties, may lead to tamoxifen-resistant tumor growth (*91-94*). This mechanism of tamoxifen resistance has been explored by quantitating and comparing the levels of tamoxifen and the various metabolites in tamoxifen-insensitive tumors and tamoxifen-sensitive tumors. Although, the conversion of tamoxifen to estrogenic or less potent anti-estrogenic metabolites can occur, the emergence of tamoxifen-stimulated growth does generally not occur in patients. Furthermore, fixed-ring derivatives of tamoxifen resulted in similar resistance in nude mice, thus excluding the role of estrogenic metabolites (*95-97*). Currently, there is no clear evidence that the pharmacology of tamoxifen explains the resistant phenotype in a significant number of patients.

ER alterations or loss

The change of ER function by a mutation or by an abnormality that exists at the mRNA or protein level represents a likely mechanism of tamoxifen resistance. Several investigators have searched for ER variants in breast cancer cell lines and breast tumor specimens. Although specific examples of ER mutations, deletions,

transitions, and RNA splice variants have been described and are present along with wild type ER in the same tumor or cell-line (*98-106*), it does not appear that alteration of the ER is the principal mechanism of resistance. Loss of ER expression may be an important step in the hormone-independent progression of some breast cancers. Most ER-negative cell lines and breast cancers lack ER mRNA as well as protein (*107*). Loss of gene transcription in the absence of mutations could be explained by epigenetic modifications that do not result in a modification of the primary DNA sequence. Methylation of CpG islands (cytosine- and guanine-rich areas) in the 5' regulatory region of genes is one such mechanism (*108*). The CpG island in the promoter and first exon of the ER gene was found to be unmethylated in normal breast tissues examined and extensively methylated in ER-negative breast cancer cells (*109*). ER gene methylation has also been detected in a fraction of ER-positive tumors. This observation suggests that heterogeneity within tumor cell populations could potentially account for ER-negative recurrent tumors arising from ER-positive tumors. Detection of ER gene methylation in primary human breast tumors suggests that this mechanism of gene silencing could contribute to the loss of ER expression and thus to hormone independence and tamoxifen resistance in breast cancer.

Alternative pathways

Tamoxifen resistance might arise from posttranslational modification of ER and/or changes in ER-regulated gene expression. Alterations, which are known to affect the transcriptional activity of ER and to enhance the agonistic activity of tamoxifen, include changes in ER-phosphorylation pathways. Tamoxifen has been reported to indirectly reduce phosphorylation of the ER via inhibition of protein kinase C (PKC) and could therefore diminish transcriptional activation (*110*). A mutation in PKC might prevent the inhibitory activity of tamoxifen resulting in the activation of estrogen-responsive genes. However, both estradiol and 4-hydroxytamoxifen generated similar ER-phosphorylation patterns (*111*), suggesting that tamoxifen does not inhibit ER phosphorylation.

The agonistic activity of the tamoxifen-ER complex was shown to be activated by the protein kinase A (PKA) pathway (*112*). Increase in the intracellular cAMP level, regulated via PKA, was shown to enhance ER phosphorylation and the transcriptional activity of the anti-estrogen-ER complex (*113*). This suggests that cross-talk between the cAMP and ER-dependent signal transduction pathways may exist. The role of cAMP-induced and tamoxifen-induced ER phosphorylation in transcriptional enhancement is complex and may involve participation of both ER-phosphorylation and phosphorylation of other factors contributing to ER-specific transcription. The transcriptional activity of the ER might also be affected by possible alterations in hormone response elements, interactions with transcriptional corepressor proteins (i.e. SMRT, NCoR) and/or coactivator proteins (i.e.AIB1, SRA, and p300) (*114-118*) and transcription factors including AP-1 (*119,120*).
Intrinsic and acquired tamoxifen resistance may also involve alterations in growth factor production and/or sensitivity (i.e. altered production of autocrine factors or paracrine interactions from adjacent ER-negative breast cancer cells or stromal cells) that affect the ER response pathway (*121-123*).

Despite the various mechanisms that have so far been examined, no clear understanding of the cause of intrinsic and acquired tamoxifen resistance in breast cancer has evolved. It has been speculated that (epi)genetic alterations in the tumor cells may be responsible for hormone-independent growth and intrinsic resistance of ER-positive tumors and contribute to development of acquired tamoxifen resistance (*122,124-127*). Dysregulation and overexpression of genes that control cell proliferation may contribute to tumor progression on tamoxifen therapy. A possible role for genes like *EGFR*, v-*Hras*, *Her2/Neu*, *FGF-4*, *TGF-β*, Cyclin D1, *Raf-1*, *IRS1*, and *IGF-1R* in hormone independence, as well as random alteration of gene expression in breast cancer cells were shown to change the hormone-dependent phenotype (*126,128-135*). Nevertheless, the involvement of these genes in tamoxifen resistance of breast cancer proved to be limited. To search for other genetic factors capable of imposing anti-estrogen resistant cell

proliferation, an *in vitro* model was generated by making use of a 'retroviral gene tagging' strategy as will be discussed in the next section.

1.3 *In vitro* model for breast cancer anti-estrogen resistance

Retroviral insertional mutagenesis

In vitro random insertional mutagenesis using replication-deficient retroviruses has proved useful to various murine model systems and *in vitro* systems for identifying a large number of genes implicated in tumorigenesis (*136*). The retroviral DNA-intermediate of retroviruses (provirus) can locally induce genetic changes by random integration in or near genes in the host genome and can profoundly affect the expression of these host genes. Proviral insertion can result in recessive mutations that disrupt and prevent expression of genes or in dominant mutations that activate gene expression.

In our laboratory this strategy has been applied for the identification of genes involved in tamoxifen resistance by the infection of estrogen-dependent breast cancer cells (human cell line ZR-75-1) with defective LN murine retrovirus resulting in random proviral insertion in the genomic DNA (*137*). Culture with tamoxifen may thus select for infected cells that acquired a tamoxifen resistant growth advantage due to the retroviral integration event in the proximity of the responsible gene. After this *in vitro* selection, the proliferating cells were expanded in a clonal fashion resulting in a total of 80 tamoxifen resistant cell lines all carrying one or more integrated LN proviral copies. On average 2 copies (range 1-10 copies) of the provirus are present in a single cell clone. Relevant viral integration sites were identified by screening for common integration sites and by making use of cell fusion-mediated gene transfer.

Identification of BCAR loci

Proviral insertion in an identical genomic locus or a 'common integration site' in hormone independent cell lines indicates the position of a relevant gene. By using the proviral genome as a tag, a common integration site in independently arisen tamoxifen-resistant cell clones can be identified and cloned with the use of

molecular biologic techniques. The panel of 80 tamoxifen resistant cell lines isolated upon retroviral infection has been used to identify common integration sites, each of which are strongly suggestive of the presence of a particular gene involved in the development of tamoxifen resistance in the proximity of the integrated provirus. Such an integration site has been termed a breast cancer anti-estrogen resistance (*BCAR*) locus (*137*). Additional evidence for the involvement of particular loci in tamoxifen resistance can be gained by cell-fusion mediated gene transfer. This requires the transfer of the genomic region that carries the BCAR locus from the lethally irradiated tamoxifen-resistant donor cells into estrogen-dependent ZR-75-1 cells. Tamoxifen-resistant cell proliferation of somatic cell hybrids containing the BCAR-locus demonstrated the dominant phenotype conferred by the locus. When more than one integration locus was present in the donor cell line, these loci could be separated among different cell hybrids and individually tested for co-segregation of particular integration loci with the tamoxifen resistant phenotype (*138*). The isolation of the responsible gene in a *BCAR* locus followed by transfection experiments demonstrating its involvement in tamoxifen resistance *in vitro*, may aid in clarifying the mechanisms involved in intrinsic and acquired tamoxifen resistance as clinically encountered. So far three *BCAR* loci have been identified that cause dominant anti-estrogen resistance in the cell line model.

BCAR1

The first common integration site, the *BCAR1* locus, was identified in six out of the panel of 80 tamoxifen resistant cell lines. Further analysis learned that these cell lines were derived from four independent viral integration events. All four proviruses were shown to have the same orientation and were located within a 2.2 kb segment of genomic DNA (Figure 2). In Chapter 2.1 the isolation and characterization of the *BCAR1* gene is described. Sequence analysis of human *BCAR1* cDNA predicted a protein of 870 amino acids that is strongly homologous to the rat gene for the p130 Crk-associated protein (p130Cas). Human BCAR1 will be termed BCAR1/p130Cas. Transfection experiments with *BCAR1/p130Cas* cDNA constructs demonstrated that this is the crucial gene in the *BCAR1* locus

involved in tamoxifen-resistant cell growth. Chapter 2.2 focuses on the clinical relevance of the BCAR1/p130Cas protein in primary breast carcinomas. Chapter 2.3 reports on the role of the BCAR1/p130Cas in acquired tamoxifen resistance. Finally, in Chapter 2.4 the localization of the BCAR1/p130Cas protein in non-malignant and malignant breast tissue is described.

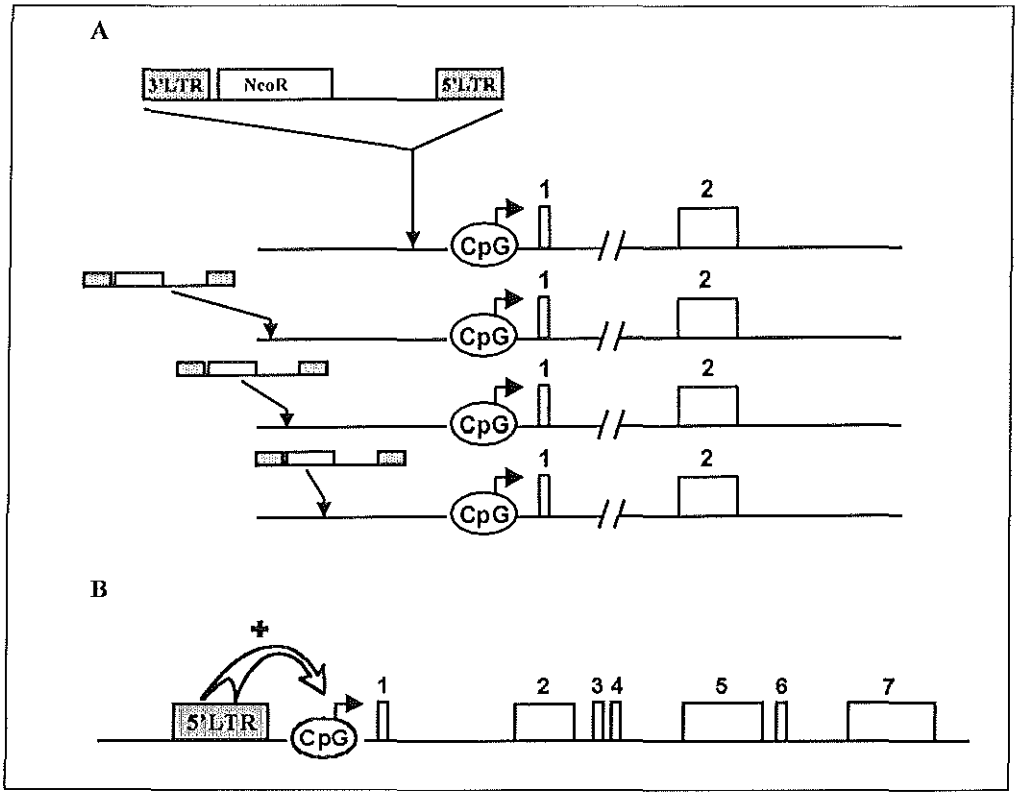

Figure 2. **Retroviral integrations in the *BCAR1* locus.**

BCAR2

The second locus implicated in tamoxifen resistance, *BCAR2*, has been identified by somatic cell fusion experiments and as a common integration site present in two independent cell lines of the panel (*138*). The proviral integrations were located within a genomic region of 14 kb. cDNA probes from the integration site recognized 5 and 8 kb mRNAs that were upregulated in BCAR2 cells compared

to parental ZR-75-1 cells. Strategies like cDNA capture, screening of testis and kidney cDNA libraries and RACE (rapid amplification of cDNA ends) have resulted in the isolation of full-length cDNA. Using *in vitro* transcription translation (ITT) a putative open reading frame (ORF) has been identified. No homology to any known sequence was found so far. Expression of this cDNA by transfection into ZR-75-1 cells is currently performed to test for the involvement of this candidate *BCAR2* gene in anti-estrogen resistance *in vitro*.

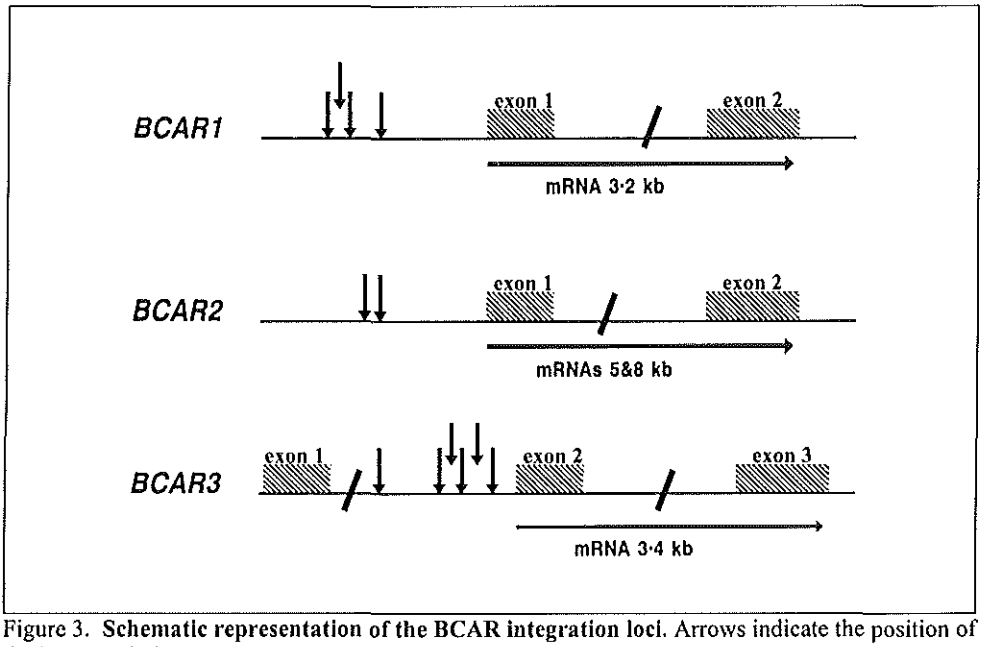

Figure 3. **Schematic representation of the BCAR integration loci.** Arrows indicate the position of the integrated viruses.

BCAR3

The third locus was identified by screening for a common integration site and was present in six independent cell lines. The integration events were located within a genomic region of 5 kb. This locus was subsequently shown to cause tamoxifen resistance following somatic cell fusion. A cDNA of 3.4 kb showing increased expression in cell lines with an integration in this locus has been isolated from a testis library. Transfection of a *BCAR3* cDNA construct into ZR-75-1 cells demonstrated anti-estrogen resistance, confirming the identity of this cDNA as

the *BCAR3* gene. Transfection of the *BCAR3* gene into another human breast cancer cell line (MCF-7) also conferred resistance of the transfectants to anti-estrogen. The *BCAR3* gene encodes a protein with an amino-terminal Src Homology 2 (SH2) domain, and a central proline/serine-rich domain that may act as SH3 interaction domain, suggesting a role for this protein in intracellular signal transduction (*139*). *BCAR3* also contains a putative Ras guanine nucleotide exchange factor (CDC25) domain at its carboxy terminus. The sequence of *BCAR3* is also published as *NSP2* which was derived from human fetal kidney (*140*). NSP2 belongs to a family of novel adapter proteins (NSP1, NSP2 and NSP3).

1.4 The p130Cas gene and encoded protein

The rat p130 Crk-associated substrate (p130Cas) was first identified as a protein hyperphosphorylated on tyrosines in v-Src and v-Crk transformed rat cells (*141*). The domain structure of the p130Cas protein consists of an amino-terminal SH3-domain, followed by a proline-rich motif, a substrate domain, and several tyrosine residues near the carboxy-terminus. The SH3 domain is known to bind to focal adhesion kinase (FAK) (*142*) and protein tyrosine phosphatase 1B (PTP1B) (*143*). The substrate domain of p130Cas encloses 15 consensus SH2 binding sites, nine of which conform to the consensus for Crk binding (*144*). Consensus binding sites also exist for the SH2 domain of Src, tensin, Abl, Grb2, PI3K, and Nck (*145*). The proline-rich region near the carboxy-terminus and tyrosine residue 762 provide the binding sites for SH3 and SH2 domains of Src kinase respectively (*144,145*).

The p130Cas protein is a member of a family of docking adapter proteins (Cas proteins) which also includes HEF1/Cas-L (human enhancer of filamentation/lymphocyte-type Cas) (*146,147*) and Efs/Sin (Embryonal fyn-associated substrate/Src-interacting or signal-integrating protein) (*148,149*). They all contain an SH3 domain in the N-terminal region, a cluster of SH2 domain-binding motifs (the substrate domain) and, with the exception of HEF1/Cas-L,

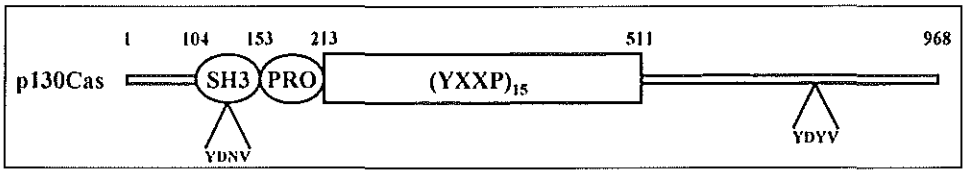

Figure 4. **Schematic representation of the structure domains of rat p130Cas.**
The aminoacids are designated by the numbers. The square box represents the substrate domain that contains multiple copies of the amino acid motif given in single letter code. YDYV represents a potential interaction site for pp60Src in the C terminus of p130Cas. *SH3*, Src-homology 3 domain; *Pro*, proline-rich stretch of amino acids.

several potential binding motifs for SH3 domains. The unique structure of these proteins indicates a role in assembling multiprotein-signaling complexes. However, for the members of this family the sequence homology and conserved domain structure may nevertheless be adapted to diverse cellular functions (*150*). The p130Cas protein is abundantly expressed in many cell types, and has been found in chicken, mouse, monkey, and human cells (*151*). The mouse homologue of rat p130Cas is very well conserved and was identified due to its capacity to specifically bind to FAK (*142*). The p130Cas protein is a cytosolic protein and can also be found in focal adhesions linking the extracellular matrix through integrins with the actin cytoskeleton of the cell (*152,153*). Evidence is accumulating, implicating p130Cas in fundamental biological processes like cellular transformation (*141,154*), cell adhesion (*155*), cell invasion and migration (*156-159*), cell spreading/cytoskeletal organization (*160,161*), growth factor stimulation (*162-164*), cytokine receptor engagement (*165,166*), and bacterial infection (*167,168*). In addition, p130Cas becomes tyrosine-phosphorylated at the SH2 binding sites in response to a number of different stimuli, many of which affect the assembly of focal adhesions and actin stress fibers. These stimuli include integrin-mediated cell adhesion (*155,169,170*), the B-cell receptor and interleukin-8 receptor (*165,166*), and stimulation of cells with nerve growth factor, bombesin and other neuropeptides, phorbol esters, bioactive lipids, and PDGF (*172*). Following oncogene-mediated cell transformation and cell adhesion, both FAK and Src have been shown to be essential for tyrosine phosphorylation

of p130Cas (*173-176*). FAK is a cytosolic protein tyrosine kinase that is critical in integrin-mediated signal transduction pathways (*177*). It is localized to focal contacts in many adherent cells in culture and becomes activated and tyrosine phosphorylated in response to cell adhesion to ECM proteins in a variety of cell types (*178*). The FAK and p130Cas proteins colocalize in focal adhesions (*142*). Tyrosine phosphorylation of p130Cas as a result of its binding to FAK is also likely to play a role in initiation of signaling resulting in cell migration. Autophosphorylated FAK recruits Src, which then phosphorylates p130Cas. The downstream pathway of the signal induced by p130Cas binding to FAK in these processes is unknown. Tyrosine phosphorylation of p130Cas is also associated with induction of migration and enhanced invasive potential of carcinoma cells *in vivo* (*157*). This migration was shown to depend on the assembly of a p130Cas/Crk adapter protein complex and p130Cas/Crk signaling may also influence tumor cell metastasis.

Following tyrosine phosphorylation, p130Cas has been shown to interact with a number of SH2-containing signaling molecules, such as the adapter proteins Crk and Nck, possibly recruiting these molecules to focal adhesions (*174,179*). Also association of p130Cas with various other proteins (FAK, RAFTK, Paxillin, Grb2, CrkL, GDNF, PTP-PEST, NSP1, AND34, and bacterial phosphatase) has been reported and appears to depend on the cell type and the process under study (*140,142,167,168,173,180-186*).

In vivo experiments suggest a crucial function for p130Cas since mouse embryos deficient for p130Cas died in utero showing poor cardiovascular development and marked growth retardation (*187*). This embryonic lethality indicates that p130Cas is important in embryogenesis, particularly in cardiovascular development, but its function in other processes may be complemented by expression of family members of p130Cas.

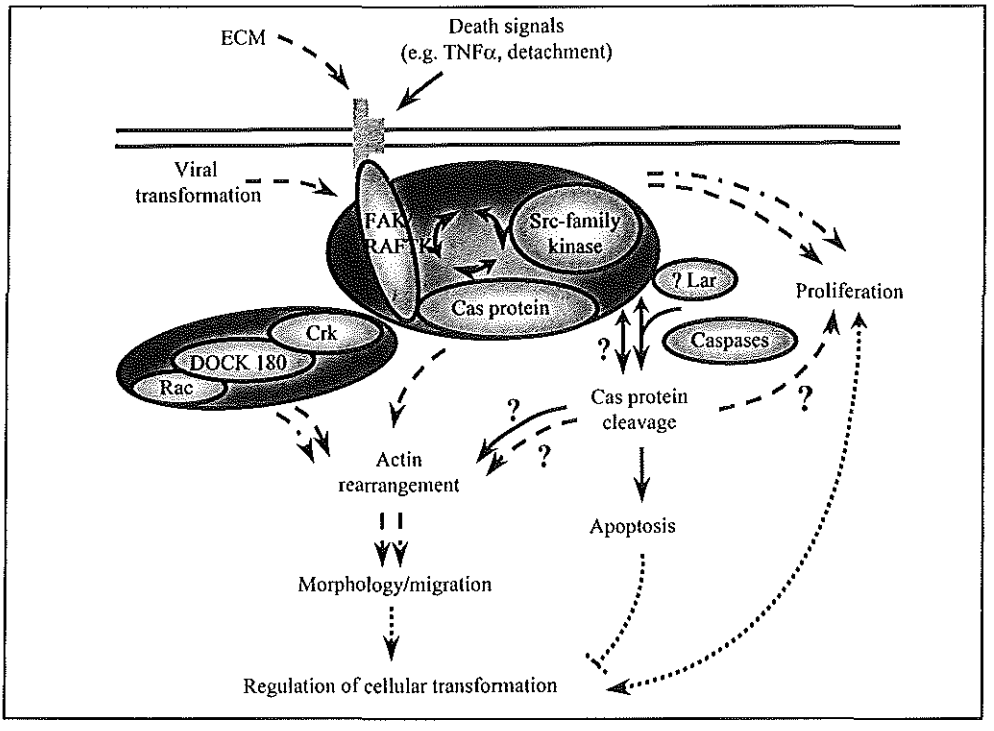

Figure 5. **Schematic representation of the interconnecting physiological pathways implicated by Cas proteins.** Signal-transduction pathways affected by viral transformation (dashed/dotted lines), integrin receptor ligation (dashed lines) and following apoptosis signals (black lines) are shown. Many external signals are transduced internally by cooperative interaction among FAK/RAFTK, Src-family kinases and Cas proteins. Upon the recruitment of Crk to this signalling complex, cells are stimulated to migrate; additionally, there is actin rearrangement as a result of signals transduced through the complex that impact both cellular morphology and migration. In response to transformation, Cas proteins appear to be constitutively tyrosine phophorylated and might play a role in the development of cancer. The formation of neoplasias might also be affected by upregulated/constitutive pro-proliferative signals via as-yet-undetermined intermediates. The downstream mediators of the different outlined pathways have not been noted as these molecules have so far not been well defined. Although it has been noted, for example, that JNK is activated following p130Cas expression and Crk recruitment, to date it is not clear what role, if any, plays in migration. Note that pathways marked with a question mark represent recently emerging potential novel effects of Cas proteins. The observation that HEF1 is cleaved in a cell-cycle related manner suggests that cleavage of this molecule might play a role in proliferation, either by direct action or by acting on an intermediary product. Pro-apoptotic signals might be transduced through the complex, potentially by destabilization of the complex through dephosphorylation, providing accessibility for caspases. Caspase cleavage could then result in the production of either pro-apoptotic Cas protein peptides or loss-of-function molecules that additionally impact on the rearrangement of the actin cytoskeleton observed during apoptosis. (reproduced from G. O'Neill *et al* (*171*))

In summary, p130Cas functions as a docking molecule associating with different adapter and effector proteins in a complex signaling network that determines the response of a cell to a particular signal. The interaction of effector proteins with p130Cas is dependent on its phosphorylation status, the external signals driving the process, the availability and affinity of the partners in the specific cell type, their activation state and the downstream cascade(s) for transmission of the signal. The majority of data on p130Cas is obtained by *in vitro* studies. However, little is known about BCAR1/p130Cas functioning in human epithelial cells and in particular in malignant breast cells.

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CHAPTER 2

Isolation, Characterization and Clinical Relevance of BCAR1

2.1

BCAR1, a human homologue of the adapter protein p130Cas, induces antiestrogen resistance in breast cancer cells

Chapter 2.1

BCAR1, a human homologue of the adapter protein p130Cas, induces antiestro resistance in breast cancer cells.

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Abstract

Background: Treatment of breast cancer with the antiestrogen tamoxifen is effective in approximately one half of patients with estrogen receptor-positive disease, but tumors recur frequently because of the development of metastases that are resistant to tamoxifen. We have previously shown that mutagenesis of human estrogen-dependent ZR-75-1 breast cancer cells by insertion of a defective retrovirus genome caused the cells to become antiestrogen resistant. In this study, we isolated and characterized the crucial gene at the Breast Cancer Antiestrogen Resistance (*BCAR*1) locus.

Methods/Results: Transfer of the *BCAR*1 locus from retrovirus-mutated, antiestrogen-resistant cells to estrogen-dependent ZR-75-1 cells by cell fusion conferred an antiestrogen resistant phenotype on the recipient cells. The complete coding sequence of *BCAR1* was isolated by use of exon-trapping and complementary DNA (cDNA) library screening. Sequence analysis of the human *BCAR1* cDNA predicted a protein of 870 amino acids that was strongly homologous to rat p130Cas adapter protein. Genomic analysis revealed that *BCAR1* consists of seven exons and is located at chromosome 16q23.1. *BCAR1* transcripts were detected in multiple human tissues and were similar in size to transcripts produced by retrovirus-mutated ZR-75-1 cells. Transfection of *BCAR1* cDNA into ZR-75-1 cells again resulted in sustained cell proliferation in the presence of antiestrogens, confirming that *BCAR1* was the responsible gene in the locus.

Conclusions: Overexpression of the BCAR1 gene confers antiestrogen resistance on human ZR-75-1 breast cancer cells. Overexpression of *BCAR1* in retrovirus-mutated cells appears to result from activation of the gene's promoter. The isolation and characterization of this gene open new avenues to elucidating mechanisms by which the growth of human breast cancer becomes independent of estrogen.

Introduction

The steroid hormone estradiol plays a pivotal role in breast cancer initiation and cell proliferation (*1,2*). Estradiol and its receptor participate in a multiprotein complex that acts as a transcription regulator for various genes (*3-6*). In approximately two thirds of all breast tumors the estrogen receptor (ER) is present and functionally active (*7-9*). The presence of the estrogen receptor in breast carcinoma cells and their responsiveness to estrogens is clinically exploited by the administration of antagonists of estrogens or antiestrogens (*10-12*). On binding, antiestrogens induce an aberrant conformation of theER, resulting in an inactive transcription complex (*13*). As a consequence the antiestrogen-receptor complex may bind DNA but can no longer modulate the expression of its target genes (*14*). This inhibition of the estrogen response pathway (*15*) may block cellular proliferation (*16-18*) and arrest tumor growth (*19,20*).

During the last two decades, antiestrogens, particularly tamoxifen, have proved to be effective in the treatment of hormone-responsive breast cancer (*21-23*). Adjuvant treatment with tamoxifen reduces tumor recurrence and increases survival of patients with ER-positive breast cancer (*24,25*). In metastatic breast cancer, tamoxifen leads to objective response in nearly one half of patients with ER-positive primary tumors (*26*). Resistance to antiestrogens, however, is a serious obstacle in the management of breast cancer. About 40% of ER-positive tumors fail to respond to antiestrogen therapy (intrinsic resistance) (*27*), whereas eventually most if not all breast tumors that initially respond to antiestrogens develop resistant metastases (acquired resistance).

The mechanisms underlying intrinsic or acquired antiestrogen resistance of breast tumors are still poorly understood. Altered pharmacology of the antiestrogens (*19,28,29*), modification of the estrogen receptor structure and function (*30-32*), and changes in the interactions between tumor cells and their environment (paracrine interactions) (*33*) have been proposed to contribute to the development of antiestrogen resistance. In addition, genetic or epigenetic changes in the tumor cells that promote the development of antiestrogen resistance have been postulated (*34,35*). Although each of these possibilities may account for or

contribute to the resistant phenotype in individual patients, none so far has been shown to explain antiestrogen resistance in a majority of patients. It is likely that progression to antiestrogen resistance is a multifactorial process.

We attempted to identify the genetic factors that may lead to antiestrogen resistance. Therefore, we applied retroviral insertion mutagenesis to the human estrogen-dependent breast cancer cell line ZR-75-1. We demonstrated that random integration of a defective murine retrovirus in the genome of ZR-75-1 cells transformed the cells from an estrogen-dependent to a tamoxifen-resistant phenotype (*36*). Mapping of common integration sites and cell fusion-mediated gene transfer experiments so far have identified three independent loci that are involved in antiestrogen resistance (*BCAR1*, *2* and *3*) (*37*). Here we report the isolation and characterization of the target gene of the *BCAR1* locus and its functional involvement in antiestrogen resistance *in vitro*.

Material and Methods

Cell Lines

The human breast carcinoma cell line ZR-75-1 and derivatives thereof were cultured as described elsewhere (*36,38*). Hybrid cell lines and *BCAR1*-transfected cell lines were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated bovine calf serum and 1nM 17β-estradiol. For *BCAR1*-transfected cell lines 500 μg/ml of the neomycin analogue G418 (Life Technologies, Breda, The Netherlands) was added to the culture medium.

The tamoxifen-resistant cell line Xl-13, with two copies of the defective LN retroviral genome (*36, 39*), one located within the *BCAR1* locus, was lethally γ irradiated (40 Gy) and subsequently fused to hygromycin B-resistant ZR-75-1 (ZH3D7) cells with polyethylene glycol as described previously (*37*). G418 plus hygromycin selection was performed in estradiol-containing culture medium (*37*).

Molecular biology techniques

A human genomic cosmid library in pJB8 (*40*) was obtained from W.J.M. van de Ven (University of Leuven, Leuven, Belgium) and screened by use of standard

protocols (*41*).

Exon trapping was performed according to the manufacturer's instructions (Life Technologies, Inc.). In short, genomic DNA of the cosmid clones HC26 and HC34 was partially digested with the enzyme *Mbo*I. Fragments of approximately 5-10 kilobases (kb) (average, 7.5 kb) were isolated and cloned in the exon-trap vector pSPL3. Batches of pooled purified plasmid DNA from five clones each were transfected into the simian kidney cell line (COS-1) (*41*). After transient expression, RNA was isolated and complementary DNA (cDNA) synthesized. Following two rounds of polymerase chain reaction (PCR), the trapped sequences were analyzed, cloned, and sequenced.

RNA was isolated from the somatic hybrid D4E5 (containing the *BCAR1* locus) and cultured for two days with 4-hydroxy-tamoxifen, by use of the CsCl-guanidine thiocyanate procedure (*41*). Poly(A) RNA was selected using the PolyATtract mRNA Isolation System (Promega Corp., Madison, WI) and converted into cDNA (Zap Express cDNA and cloning kit, Stratagene Cloning Systems, La Jolla, CA) as recommended by the manufacturer. A cDNA library of approximately $2x10^6$ phages was created and screened. Twenty-one phage clones hybridizing with a *BCAR1* exon-trap probe were isolated and sequenced by use of standard procedures (*41*). DNA and protein sequence analyses and alignments were performed using DNASIS (Hitachi Software Engineering America Ltd, Brisbane, CA), Blast (www.ncbi.nlm.gov/blast), and Clustalw (www2.ebi.ac.uk/clustalw). The *BCAR1* sequence has been assigned Genbank Accession No. AJ 242987.

Northern blots were prepared by use of 1% agarose-formaldehyde gels as described previously (*41*). Blots were hybridized with random-primer labeled probes for *BCAR1*, the estrogen-regulated PS2 gene, and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene for loading control as described (*42,43*)

Mapping of the *BCAR1* locus was performed on genomic DNA from a panel of 30 somatic cell hybrids obtained from D.F. Callen (Women's and Children's Hospital, Adelaide, Australia). PCR was performed with the locus-specific primers 1140 5'-CCCCACATACCCAGCACA-3' and 1142 5'-

CCCAGCTCCTCCTTATTCA-3'. The amplified product was verified by hybridization with the *BCAR1* locus-specific probe 18/1 (*36*).

Expression constructs and transfection of BCAR1 in ZR-75-1 cells

The full-length *BCAR1* cDNA was inserted into the *Eco*RI-*Xho*I site of the long terminal repeat-promoted pLXSN expression vector (*39*) and into a modified version of the episomal vector LZRS-IRES-Neo (LZRSpBMN-IRES) (*44*). In the latter vector, the *BCAR1* cDNA and the neomycin resistance gene (to confer selectability) were separated by an IRES sequence, so that both genes were under control of a single long terminal repeat (LTR) promoter and transcribed as a polycistronic messenger.

Stable transfectants were generated with the pLXSN/*BCAR1* construct by use of calcium phosphate precipitation (*41*). Transfection of ZR-75-1 cells with the episomal LZRSpBMN-IRES/*BCAR1* vector construct was performed by ue of Lipofectin reagent (Life Technologies, Inc.), according to the manufacturer's instructions. Transfectants were selected for neomycin resistance (500 μg of G418/ml) in estradiol-containing medium.

Proliferation assay

Somatic hybrid cells, *BCAR1* and mock transfected cells were grown in culture medium supplemented with 1nM estradiol. Cells were harvested at 80% confluency by trypsinization. The cells (0.7×10^6) were seeded in 25-cm^2 flasks, in triplicate, and grown in the presence of either 1 μM OH-Tamoxifen or 100 nM of the pure antiestrogen ICI 182,780 (Zeneca Pharma, Ridderkerk, The Netherlands). After 4-6 days, the cells were again trypsinized, counted using a Coulter Z1 cell counter (Coulter Electronics Ltd, Luton, UK), and re-seeded in triplicate as above. To follow the proliferation of the cells, the procedure was repeated at several times points and for each cell line. In case of limited recovery of cells at the end of the assay period, only two flasks were re-seeded or the culture was terminated. Proliferation was determined as the fold multiplication

(mean of at least two flasks) relative to day 0.

Drug-sensitivity tests on ZR-75-1-derived cell lines were performed in complete medium containing estradiol in 96-well plates. Five thousand cells were plated with threefold dilutions (four wells each) of the drug. After 9 days of culture in the presence of the drug, viable cells were measured by use of the MTT assay as described (*38*)

BCAR1 protein detection

BCAR1 and mock transfected cells were trypsinized, rinsed with phosphate buffered saline (PBS), sonicated for 10 seconds, and lysed for 10 minutes at 100 °C in 1% sodium dodecyl sulphate (SDS), 10 mM Tris-HCL pH 7.5. The protein concentration of the crude lysates was determined (BCA™, Protein assay reagent, Pierce Chemical Co., Rockford, IL). Equal amounts (3.3µg) of protein were used for SDS-PAGE (8% acrylamide) and subjected to Western blot analysis as described (Van der Flier et al., accompanying paper). BCAR1 was visualized with the mouse monoclonal antibody to rat p130Cas (Transductional Laboratories, Lexington, KY). In addition, polyclonal antibodies raised against the N-terminus and C-terminus of rat p130Cas (Cas N-17 and Cas C-20, Santa Cruz Biotechnology Inc.,Santa Cruz, CA) were used to identify BCAR1 in an independent control experiment.

Results

Transfer of the BCAR1 locus by cell fusion

We have used retrovirus insertion-mediated mutagenesis on the human estrogen-dependent breast cancer cell line ZR-75-1 to generate eighty cell lines showing resistance to 4-hydroxy-tamoxifen (*36*). Integration of the defective murine retrovirus (LN) into the host cell genome could transform these cells to a tamoxifen-resistant phenotype. With the use of integration site-specific probes, we previously found a common integration locus for the retrovirus in a subset of this panel of cell lines. Four independent cell clones were identified by Southern blot analysis with a viral integration in the same orientation at different positions

within a 2.2-kb genomic region (*36*) (see also Fig. 2A). We termed this locus *BCAR1*, for breast cancer antiestrogen resistance locus — i.e., the first *BCAR* locus identified.

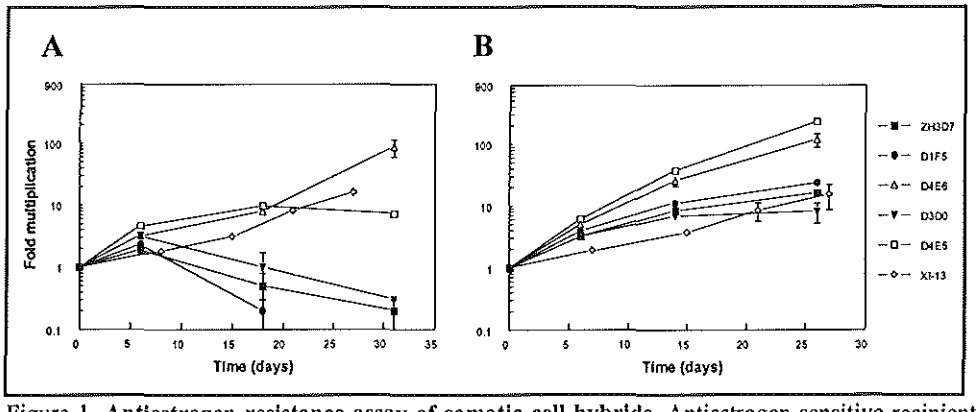

Figure 1. **Antiestrogen resistance assay of somatic cell hybrids.** Antiestrogen-sensitive recipient cells (ZH3D7), antiestrogen-resistant donor cells (XI-13), and somatic cell hybrids with (D4E5 and D4E6) and without (D1F5 and D3D0) *BCAR1* locus were cultured in triplicate in serum-containing medium with 100nM of ICI 182,780 (panel A) or without (panel B). At the times indicated, cells were harvested, counted, and re-seeded in triplicate. The numbers of cells recovered under antiestrogen at day 18 were insufficient to continue the culture of D1F5 and only allowed for reseeding in duplicate for ZH3D7and D3D0 (panel A). Culture of the steadily growing XI-13 cells under antiestrogen was terminated at day 27 (panel A). Indicated is the fold multiplication (mean of at least two flasks ± standard deviation) relative to day 0. Error bars were smaller than symbols in cases where they do not appear.

To establish whether retroviral integration in the *BCAR1* locus induces tamoxifen resistance in ZR-75-1 cells, we transferred the genomic region encompassing the *BCAR1* locus from tamoxifen-resistant cells into estrogen-dependent ZR-75-1 cells by cell fusion. XI-13 cells carry two copies of the defective retroviral genome, one copy of which is located within the *BCAR1* locus.

On lethal γ irradiation, the cells were fused to hygromycin B-resistant ZR-75-1 cells (ZH3D7) with polyethylene glycol as described previously (*37*). The neomycin resistance gene in the integrated retrovirus allowed the isolation of somatic-cell hybrids by dual selection with G418 plus hygromycin in estradiol-containing culture medium. Twelve cell hybrids containing either one of the

integration loci in the ZR-75-1 background were rescued and analyzed. Southern blotting analysis using a specific probe for the neomycin resistance gene revealed that five hybrids had retained the *BCAR1* integration locus (a 15 kb *Bgl*II fragment), whereas the remaining seven carried the other integration locus (a 10 kb *Bgl*II fragment). Both the virus-induced *BCAR1* cell line XI-13 and the *BCAR1* locus-containing cell hybrids derived therefrom (e.g., D4E5 and D4E6) showed enhanced proliferation when cultured in the presence of the antiestrogen ICI 182,780 (Fig. 1A). Hybrid cell lines carrying the other locus, as well as the ZH3D7 recipient cells, were growth inhibited. In the absence of antiestrogens, *BCAR1* locus-containing hybrids also demonstrated increased proliferation compared to the control cell lines (Fig. 1B). Note that the proliferation of most cell lines is more efficient in the absence of antiestrogen, which is likely because of some residual estrogens in the serum-containing medium. In the presence of antiestrogens the ER-pathway is more effectively blocked. The lack of the estrogen receptor in XI-13 cells (*36*) can explain the comparable growth levels of these cells in presence as well as in absence of antiestrogens (Fig. 1). These fusion experiments demonstrated unambiguously that the *BCAR1* locus induced antiestrogen-resistant proliferation of the somatic cell hybrids, and that the BCAR1 phenotype is dominant.

Cloning of the BCAR1 locus

A human cosmid library was screened with genomic probes that have been employed to identify the *BCAR1* locus (*36*). The probes included a 500 basepair (bp) *Apa*LI-*Pst*I fragment (14B2) and a 450-bp *Sph*I fragment (18/1). Both probes reside at the center of the viral integration site in the *BCAR1* locus (Fig. 2A). Cosmids were isolated and analyzed by restriction mapping, resulting in a continuous contig of approximately 80 kb (Fig. 2A). Unique probes derived from the 80-kb contig identified no further proviral integrations events in the 80 antiestrogen resistant cell lines from our panel.

Identification of the BCAR1 gene

Two of 10 overlapping cosmid clones covering the major part of the *BCAR1* locus—HC26 and HC34—were selected for identification of transcripts by exon trapping. A total of 31 trapped sequences were recovered, of which 16 were related to known genes. Eight clones corresponded to exons 2-7 of the chymotrypsin gene (Accession number NM_001906), and the remaining eight clones appeared to be homologous to sequences of the rat p130Cas gene (Accession number D29766) (*46*). Of the remaining 15 clones, two seemed to be generated because of cryptic splicing events in frequently occurring alu-type

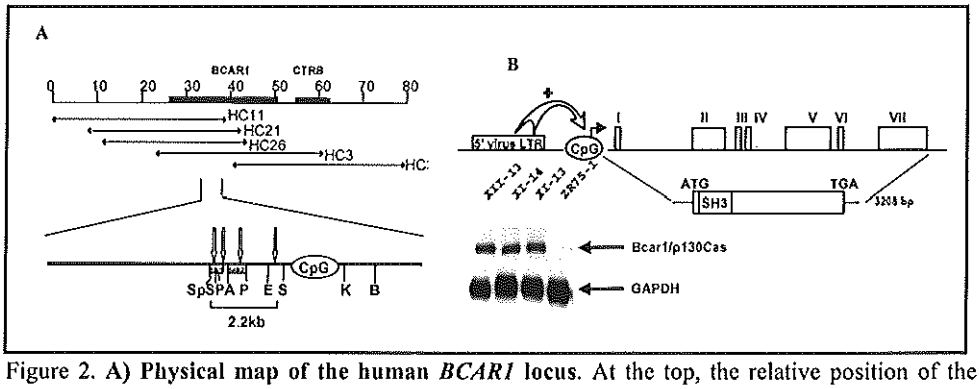

Figure 2. **A) Physical map of the human *BCAR1* locus.** At the top, the relative position of the *BCAR1* gene and part of the chymotrypsin gene (CTRB) is shown (black bars) at a scale in kilobases kb). The positions are shown of five representative overlapping cosmid clones, forming a continuous contig of 80kb. At the bottom is an exploded view of the 2.2-kb integration region. Arrows indicate integration sites in various tamoxifen-resistant BCAR1 cell lines. Note that each arrow represents an integration event in an individual BCAR1 cell line. The *BCAR1* locus-specific probes 18/1 and 14B2 are shown as shaded boxes. (Sp=*Sph*I, S=*Sst*I, P=*Pst*I, A=*Apa*LI, E=*Eco*RI, K=*Kpn*I, and B=*Bam*HI). **B) Schematic representation of the *BCAR1* gene (not to scale).** Shown are the relative positions of the 5' viral LTR, a putative CpG island, and the complete *BCAR1* gene consisting of seven exons. Underneath, the *BCAR1* complementaryDNA is drawn encompassing an 870 amino acid open-reading frame with an SH3 domain in the N-terminal part. The insert on the left presents a Northern blot containing approximately 3 µg of polyA messengerRNA of three representative BCAR1 antiestrogen-resistant cell clones raised by retroviral insertion mutagenesis (XII-13, XI-14, and XI-13) and ZR-75-1 cells. The blot was hybridized with trapped sequences homologous to rat p130Cas (top panel) or with GAPDH probe to control for loading (bottom panel). Blots were washed until 0.3x standard saline citrate (SSC) at 65°C for 30 min.

repetitive sequences in the *BCAR1* locus, while the other did not show homology to any known sequences in available databases.

The trapped exons were hybridized to polyA messenger RNAs (mRNAs) isolated from the antiestrogen-resistant *BCAR1* cell lines (XII-13, XI-14, and XI-13) and from the parental cell line ZR-75-1. The chymotrypsin gene sequences did not hybridize in a Northern blot analysis, indicating that this gene is not expressed in our ZR-75-1-derived cell lines. In contrast, exon sequences that were homologous to the rat p130Cas gene hybridized to a single mRNA of 3.2kb (Fig. 2B). This transcript appeared to be increased in the antiestrogen-resistant cell lines but not in the parental cell line. As shown in Fig. 2B, ZR-75-1 exhibits only low levels of an equally sized transcript. The differential expression of this transcript suggested that the rat p130Cas-homologous gene was the candidate breast cancer antiestrogen resistance gene, *BCAR1*. Conclusive evidence for its role in antiestrogen resistance required the isolation of the *BCAR1* cDNA and its functional transfer to ZR-75-1 cells by transfection (see below).

Primary structure of the BCAR1 gene

A cDNA library from the antiestrogen-resistant *BCAR1* cell hybrid D4E5 was screened with p130Cas homologous sequences retrieved from exon trapping. Twenty-one positive cDNA clones were identified and assembled by sequence alignment into a continuous cDNA of 3208 bp. The *BCAR1* cDNA encloses an open reading frame of 2610 nucleotides. At position 122, an initiation codon is flanked by sequences matching Kozak's criteria (*47*). The open reading frame is flanked at the 3' end by a translation termination codon (TGA) at position 2732 and a 3' untranslated sequence of 468 nucleotides that contains multiple stop codons. A canonic polyadenylation site is located 14 bp in front of the start of the polyA tail in the cDNAs. The Genbank Accession Number for the *BCAR1* sequence is AJ 242987.The open-reading frame has a coding capacity for a protein of 870 amino acid residues, with a calculated molecular mass of approximately 93 kD. The predicted protein features an Src Homology 3 (SH3) domain in the N-

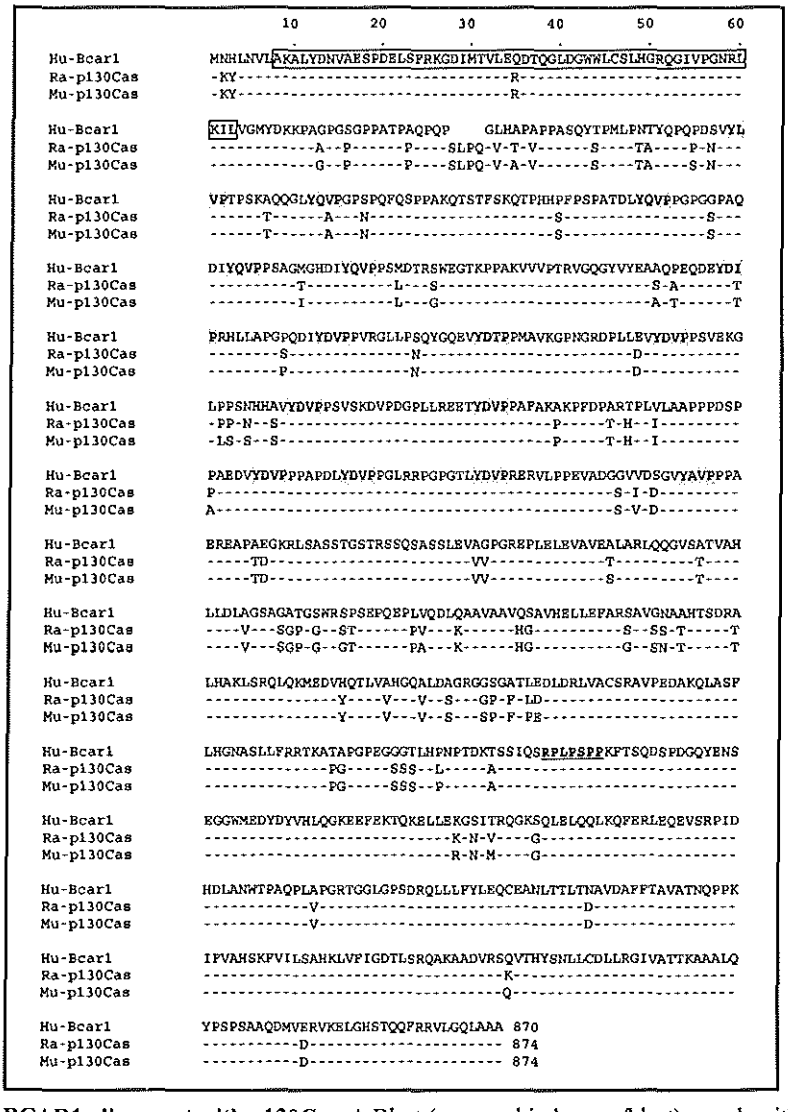

Figure 3. **BCAR1 alignment with p130Cas.** A Blast (www.ncbi.nlm.gov/blast) search with *BCAR1* complementaryDNA sequences in available databases demonstrated homology to rat and murine p130Cas sequences. An alignment is shown of the predicted protein sequences of human BCAR1 with rat and mouse p130Cas. A single gap of four amino acid residues has been introduced by the Clustalw program (www2.ebi.ac.uk/clustalw) for optimal alignment. An SH3 homology domain (boxed), a proline-rich sequence region (bold and underlined) and multiple potential tyrosine hosphorylation sites (shaded) are indicated. The *BCAR1* sequence has been assigned Genbank Accession No. AJ 242987.

terminal part and multiple potential tyrosine phosphorylation sites (Tyr-Ala/Pro-Xxx-Pro) in the central part of the protein (Fig. 3). The C-terminal part encloses a proline-rich stretch (Arg-Pro-Leu-Pro-Ser-Pro-Pro) that may interact with the SH3 binding site of the Src protein (*48*). Sequence alignment of BCAR1 revealed extensive homology (91%) with rat and mouse p130Cas protein (Fig. 3).

When hybridized with *BCAR1* cDNA probes, RNA preparations of BCAR1 cell lines displayed a single transcript of approximately 3.2 kb (Fig. 2B). The size of the mRNA is in agreement with that of the cDNA, suggesting that the cDNA represents the full-length transcript. Northern blots of mRNAs derived from a panel of multiple human tissues hybridized to the *BCAR1* cDNA probe showed that the *BCAR1* transcript is widely expressed (Fig. 4). The 3.2-kb mRNA was present in all tissues tested, being most abundant in testis and with only a low representation in liver, thymus, and peripheral blood leukocytes.

Additionally, *in vitro* transcription and translation of the *BCAR1* cDNA demonstrated that the cDNA encodes a protein with an apparent molecular mass of approximately 116 kD on SDS-PAGE. The discrepancy in observed and calculated (93-kD) molecular mass is probably because of folding of the molecule, causing aberrant gel mobility. Both rat p130Cas and the BCAR1 protein exhibit the same size. As expected, a monoclonal antibody directed against rat p130Cas identified the BCAR1 gene product (Fig. 5A) as well as polyclonal antibodies that are directed against either the N- or C-terminus of the rat p130Cas (data not shown).

Genomic organization of BCAR1

The genomic organization of the *BCAR1* gene was delineated by use of the *BCAR1* cDNA as a template. Restriction mapping and partial sequencing of cosmid clones HC26 and HC3 revealed that the transcribed region of the *BCAR1* gene consists of seven exons (I-VII) spanning approximately 25kb of genomic DNA (Fig 2B). The size of the exons varies from 76 to 1101 bp and the size of the introns from 182 bp to approximately 8.5 kb. Sequencing through the exon-intron boundaries demonstrated that all splice junctions conform to the GT/AG rule for

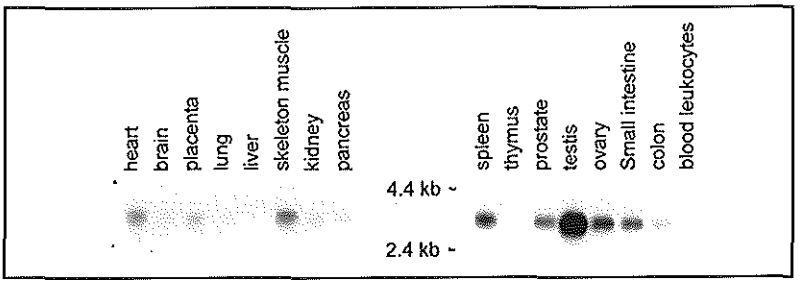

Figure 4. **Expression of *BCAR1* in various human tissues.** Commercially available Northern blots containing polyA messengerRNA (2μg) of multiple human tissues (MTN blots; Clontech Laboratories, Inc, Palo Alto, CA) and checked for loading by the supplier were hybridized with a labeled *BCAR1* total complementaryDNA, washed until 0.3xSSC at 65°C for 30 minutes, and exposed to an x-ray film for 4 days at –70 °C with an intensifying screen. Size markers are indicated.

exon/intron junctions. Southern blot analysis showed that all restriction fragments from the cosmid clones that hybridized to *BCAR1* cDNA were also present in genomic DNA. More important, the coding sequence of the *BCAR1* gene was found identical to that of the cDNA, excluding cloning artifacts.

Exon 1 of *BCAR1* contained the first 12 bp of the coding sequence. It is of interest that this position of the exon-intron boundary exactly matched the position where alternative splicing has been observed in rat as well as mouse p130Cas (*46,49*). Exons 2-6 all mapped within the coding sequence, and exon 7 contained the last 509 coding nucleotides and the complete 3' nontranslated region. The 5' flanking region of the *BCAR1* gene had a high G+C content (80%) and lacks suppression of CpG dinucleotides, which is characteristic for CpG islands. Such a configuration, which is often found for housekeeping genes, is consistent with the ubiquitous expression of the *BCAR1* gene (Fig. 4).

In situ hybridization with the complete cosmid HC26 suggested that the *BCAR1* gene was located at human chromosome 16q22-23 (Van Agthoven T.: personal communication). Primers specific for the integration region were used to analyze

a panel of radiation hybrids containing fragments of human chromosome 16. PCR analysis on genomic DNA derived from these hybrids cell lines determined the position of the *BCAR1* locus to 16q23.1. Hybrids lacking the region 16q23.2 to telomere (e.g., CY145) gave no *BCAR1*-specific product, whereas hybrids that retained the 16q23.1 region (e.g., CY116 and CY117) gave specific products (*50*). Evidence for this position of the *BCAR1* gene was further supported by the concurrent trapping of exons of the chymotrypsin gene that had been assigned to chromosome 16q23.1 (*50*). Hybridization with chymotrypsin gene probes on the *BCAR1* cosmid clones confirmed the position of the chymotrypsin gene 4-5 kb downstream of the *BCAR1* gene (Fig. 2A).

Considering the vast homology of the *BCAR1* cDNA with rat and murine p130Cas sequences and the strict conservation of the first exon-intron boundary, we postulate that the *BCAR1* gene is the human p130Cas homologue, and we will further refer to this gene as: *BCAR1/p130Cas*.

Transfection of BCAR1/p130Cas cDNA and estrogen independence in ZR-75-1 cells

To demonstrate that BCAR1/p130Cas is functionally involved in antiestrogen resistance, the full-length *BCAR1/p130Cas* cDNA was introduced in antiestrogen-sensitive ZR-75-1 cells by transfection. Five independent LXSN / BCAR1 / p130Cas transfectants were generated, carrying an intact transgene integrated in the host genome as determined by Southern blot analysis. Similarly, eight independent cell lines carrying the *BCAR1/p130Cas* cDNA in an episomal vector were established. In addition, more than 22 vector-control cell lines have been raised.

All transfected cell lines were generated in estrogen-containing medium and subsequently tested for proliferation in the presence of either 4-hydroxy-tamoxifen or the pure antiestrogen ICI 182,780. A typical example of such an assay using 4-hydroxy-tamoxifen is shown in Fig. 5A. Aliquots of the cultured cells have been used to measure the amount of BCAR1/p130Cas protein with the use of Western blotting and immunodetection (Fig. 5A). All cell lines exhibiting

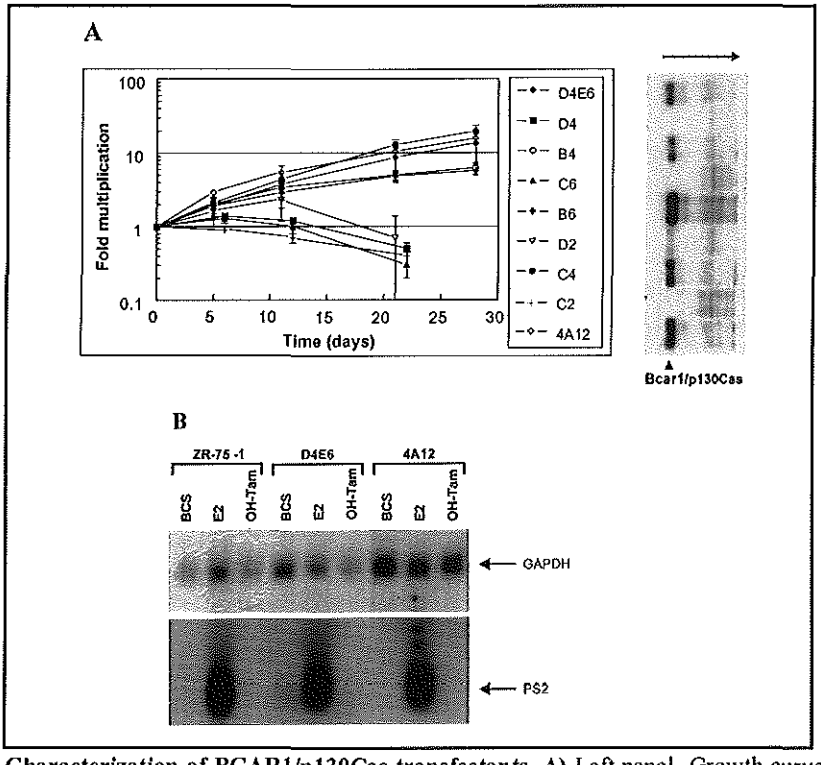

Figure 5. **Characterization of BCAR1/p130Cas transfectants.** A) Left panel- Growth curves of a representative selection of BCAR1-transfected cell lines grown in presence of antiestrogen. Three flasks of each LXSN/BCAR1/p130Cas-transfected cell line (4A12, B4, B6, and C4), the somatic cell hybrid (D4E6) and vector-only control cell lines (C2, C6, D2, and D4) were cultured in presence of OH-tamoxifen (OH-Tam) (1μM). At indicated time points, three flasks were harvested, processed for cell counting, and reseeded. The number of cells recovered for C2 only allowed for reseeding in duplicate for the last time point. Indicated is the fold multiplication (mean of at least two flasks ± standard deviation) relative to day 0 (see Fig 1). Right panel- A typical Western blot of cell lysates (3.3 μg protein) separated on SDS-Page (8%), transferred to nitrocellulose filters, and visualized with mouse monoclonal antibody to rat p130Cas. The blot contained cell lysates isolated from these cell lines at day 12/13. An arrow indicates the direction of migration in SDS-PAGE gels, and an arrowhead marks the position of BCAR1/p130Cas. Similar expression levels were observed on day 0 and day 5/6. B) RNA was prepared from the indicated cell lines after culture in bovine calf serum-containing medium (BCS) supplemented with 1nM of estradiol (E2) or 1μM of OH-Tam for 5 days. Approximately 5μg of RNA was blotted and hybridized with probes for GAPDH and pS2. Blots were washed until 0.3xSSC at 65°C for 30 min.

high expression of BCAR1/p130Cas (e.g., 4A12, B4, B6, C4 and the somatic cell hybrid D4E6) showed net proliferation in the presence of 4-hydroxy-tamoxifen. In contrast, ZR-75-1 and all vector control cell lines (e.g., C2, C6, D2, and D4) expressed very low levels of or no BCAR1/p130Cas and failed to proliferate in the presence of the antiestrogen. Similar results were obtained in the assays by the use of the pure antiestrogen ICI 182,780 (data not shown). Of interest, BCAR1/p130Cas-transfected cells underwent a morphologic change when cultured in the presence of antiestrogens (data not shown). The cells displayed a typical flattened shape with multiple protrusions (similar to virus-induced cell lines), which may be suggestive of a reorganization of the cytoskeleton. Further experiments with the use of immunofluorescence are needed to verify these observations. To investigate the possibility that BCAR1/p130Cas-overexpression also generates resistance to other drugs, we performed cell survival assays using doxorubicin, 5-fluorouacil and methotrexate. These pilot expriments showed no relation between sensitivity to these drugs and overexpression of BCAR1/p130Cas; i.e., comparable ID_{50} (dose showing 50% inhibition) values were obtained for expressing cell lines and cell lines without overexpression (data not shown). Northern blotting analysis of BCAR1/p130Cas-transfected cells grown under antiestrogen selection revealed an impaired expression of the estrogen-regulated

PS2 gene (*43*) similar to the parental cells (Fig. 5B), suggesting that the proliferation of these cells was independent of ERfunction. Because integrin and growth factor-receptor pathways may signal through p130Cas to the mitogen-activated protein kinase (MAPK) pathways (*51-54*), we have performed preliminary experiments to monitor the activation state of various MAPKs in BCAR1/p130Cas-transfected cell lines. Specific antibodies directed against activated MAPKs (ERK, p38, and JNK) failed to document constitutive activation of these kinases in the presence of antiestrogens (not shown). Because of the slow growth and response kinetics of the transfected cells in antiestrogen-supplemented cultures, the levels of activated MAPK may have been below the threshold of detection in our tests.

Discussion

The mechanisms underlying antiestrogen resistance in breast cancer are the subject of intensive study. Our working hypothesis was based on the assumption that genetic or epigenetic alterations in the tumor cells contribute to the failure of the therapy. To identify target genes, we have employed a model system of estrogen dependence resembling that seen in human breast cancer. In this article, we describe the cloning and characterization of the *BCAR1* gene. On the basis of our findings, *BCAR1* appears to be the human homologue of the rat and mouse p130Cas genes; their function is described below. Overexpression of BCAR1/p130Cas in human estrogen-dependent ZR-75-1 breast cancer cells is sufficient to drive cell proliferation in the presence of antiestrogens. No difference in structure or size of the *BCAR1/p130Cas* transcript was seen in the virus-infected cell lines. This suggests that overexpression of the *BCAR1/p130Cas* gene is achieved by increased activity of the *BCAR1/p130Cas* gene promoter, likely due to the presence of strong enhancer elements within the viral LTRs (*55*). This mechanism of transcription enhancement is different from the observed viral promoter-insertion activation of the BCAR3 gene that resulted in an altered BCAR3 transcript and protein (*43*). It is possible that such events are selected against, in the case of the *BCAR1/p130Cas* gene, to ensure an intact and functional protein.

Our experiments indicate that BCAR1/p130Cas-mediated antiestrogen resistance is not associated with resistance to other drugs relevant to treatment of breast cancer. In addition, the ER does not play a crucial role in the resistant phenotype. Cell lines obtained after viral integration in the *BCAR1* locus (e.g., XI-13) completely lack ER expression and function (*36*) and show comparable growth rates in the absence or presence of antiestrogens (Fig. 1). The somatic cell hybrids containing the *BCAR1* locus and the BCAR1/p130Cas transfectants are estrogen responsive with respect to growth and regulation of PS2 expression. These variants exhibit antiestrogen-resistant cell proliferation but are not dependent on antiestrogen for growth (Fig. 1B). These observations suggest a cell proliferation

regulatory mechanism involving BCAR1/p130Cas, which bypasses the ER-regulated pathway.

The observation that the *BCAR1* gene is highly homologous to the well-characterized rat and mouse p130Cas genes may help to understand the mechanism of antiestrogen-resistant breast cancer cell proliferation. Rat p130Cas has been identified as the major tyrosine-phosphorylated protein in v-Src and v-Crk transformed rat cells (*46,56*). Subsequent reports have implicated p130Cas in various processes in different cell types, including cell transformation (*57,58*), linking the extracellular matrix with the actin cytoskeleton (*59,60*), integrin signaling (*51,61-64*), growth factor receptor signaling (*53,54,65-68*), antigen receptor signaling (*69*), cell migration and invasion (*70-72*), bacterial invasion (*73,74*), and essential cardiovascular development (*58*). This varied role of p130Cas may be explained by the presence of several protein–protein interaction domains (*46,75*), which is the hallmark of this novel class of adapter proteins. Family members are HEF1 (also designated CasL) (*76,77*) and Efs (also termed Sin) (*78,79*), which may have distinct functions (*80*). The SH3 domain, in particular, is well conserved between these family members and has been shown to interact with proline-rich target sequences from FAK (*49*), RAFTK (*81*), PTP-PEST (*82*), CAKbeta (*83*) and PTP1B (*52*). The central part of p130Cas contains multiple potential tyrosine-phosphorylation sites capable of interacting with SH2 domain-containing proteins like Crk (*84,85*), Src (*48*) and Nck (*51*). The C-terminal part of the p130Cas family members is well conserved but has not yet been implicated in a particular function in mammalian cells. Together, the numerous documented interactions and functions of p130Cas in various biologic processes and in different cell types suggests a dynamic role for p130Cas. The outcome of these complex interactions will depend on the availability of binding partners (including their activation status and affinity) and the specific process in that cell type.

So far, little information is available regarding the role of BCAR1/p130Cas in regulation of proliferation of breast epithelial cells. The observation that expression of BCAR1/p130Cas in primary breast cancer predicted disease

prognosis and response to tamoxifen therapy (*45*) suggests a genuine role for BCAR1/p130Cas in growth control of breast cancer cells. Further elucidation of the pathway involved in BCAR1/p130Cas-mediated cell proliferation in breast cancer cells may identify *in vitro* key regulators and possibly novel clinical targets. The recognition that both BCAR1/p130Cas and BCAR3 (*42*) can control proliferation of breast cancer cells further supports the validity of our model system to identify breast cancer antiestrogen resistant genes.

Notes

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2.2

BCAR1/p130Cas and primary breast cancer: prognosis and response to tamoxifen treatment

Chapter 2.2

Bcar1/p130Cas and Primary Breast Cancer: Prognosis and Response to Tamoxifen Treatment

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Abstract

Background: The product of the Bcar1/p130Cas gene causes resistance to antiestrogen drugs in human breast cancer cells *in vitro*. The levels of Bcar1/p130Cas protein were determined in a large series of primary breast carcinomas. *Methods*: We measured Bcar1/p130Cas protein in cytosol extracts from 937 primary breast carcinomas by Western blot analysis. The levels of Bcar1/p130Cas protein were tested for associations and trends against clinicopathologic and patient characteristics, the lengths of relapse-free and overall survival (n=775), and the efficacy of first-line treatment with tamoxifen for recurrent or metastatic disease (n=268). *Results*: Bcar1/p130Cas levels in primary tumors were associated with age/menopausal status and the levels of estrogen receptor and progesterone receptor. In univariate survival analysis, higher Bcar1/p130Cas levels were associated with poor relapse-free and overall survival (both two-sided $P = .04$; log-rank test for trend). In multivariate analysis, a high level of Bcar1/p130Cas was independently associated with poor relapse-free and overall survival. The response to tamoxifen therapy in patients with recurrent disease was reduced in patients with primary tumors that expressed high levels of Bcar1/p130Cas. In multivariate analysis for response, Bcar1/p130Cas was independent of classical predictive factors, such as estrogen receptor status, age/menopausal status, disease-free interval, and dominant site of relapse. *Conclusion*: Patients with primary breast tumors expressing a high level of Bcar1/p130Cas protein appear to experience more rapid disease recurrence and have a greater risk of (intrinsic) resistance to tamoxifen therapy. Thus, measurement of Bcar1/p130Cas may provide useful prognostic information for patients with primary or metastatic breast cancer.

Introduction

The breast epithelium is continuously exposed to the steroid hormone estrogen, which has been implicated in the proliferation of breast tumor cells and in the progression of breast cancer. Estrogens act by binding to the nuclear estrogen receptor (ER) present in the breast epithelial cells. The ER-ligand complex

specifically activates target genes, resulting in a cascade of events leading to cell proliferation and differentiation (*1,2*). The recent cloning of a closely related ER, ERβ (*3,4*), suggests the presence of alternative pathways of estrogen signaling. As yet, little is known about the role of ERβ in the development of human breast cancer (*5*).

Endocrine therapies have been developed to block the effects of estrogen on cancer cells or to reduce serum estrogen levels. Tamoxifen is an antiestrogenic compound that is widely used as an adjuvant treatment for patients with ER-positive breast tumors (*6*). The decision to use endocrine therapy for advanced breast cancer is based on a number of predictive factors, including ER or progesterone receptor (PgR) status, age, menopausal status, disease-free interval, and site of relapse. About 50% of patients with advanced breast cancer receiving endocrine treatment have an objective response or stable disease (no change > 6 months). The rest of the patients have a short period of stable disease or immediate progressive disease (*7*). The latter patients are considered to have intrinsic-resistant disease. However, almost all responding patients eventually experience disease recurrence (i.e., acquired resistance). Tamoxifen resistance may result from multiple mechanisms, and a number of causes have been suggested, including (epi)genetic alterations, resulting in ER mutants and splice variants (*8-10*), and alteration of absorption, distribution, and metabolism of tamoxifen (*11,12*). Elucidation of the mechanism(s) of tamoxifen resistance is, therefore, a comprehensive challenge in cancer biology.

To identify genes involved in hormone-independent growth, we used the technique of retroviral insertional mutagenesis and ZR-75-1 cells, a human breast cancer cell line. With this system, we have identified three loci responsible for breast cancer antiestrogen resistance (BCAR) (*13-15*). The first genomic locus linked to the antiestrogen drug-resistant phenotype of the cells that we identified was the BCAR1 locus (*13*). Characterization of the BCAR1 gene indicated that it is the human homologue of the rat-docking protein also known as p130 Crk-associated substrate (p130Cas) (*16*). p130Cas, which contains an homology (SH) 3 domain, a proline-rich region, and a substrate domain containing multiple SH2-

binding motifs, was identified as a major substrate for protein tyrosine phosphorylation in v-Src- and v-Crk-transformed cells (*17,18*) and has been implicated in a variety of biologic processes, including cell adhesion (*19,20*), cell migration (*21-23*), growth factor stimulation (*24*), cytokine receptor engagement (*25*), and bacterial infection (*26,27*). Cell fusion-mediated transfer of the BCAR1 locus and transfection of BCAR1/p130Cas complementary DNA into ZR-75-1 cells demonstrated that overexpression of Bcar1/p130Cas results in sustained cell proliferation in the presence of antiestrogens (*16*). These observations prompted us to investigate the clinical relevance of Bcar1/p130Cas expression by measuring Bcar1/p130Cas protein in specimens of human breast carcinoma and testing these data against various characteristics of the patients and their disease for clinically relevant associations.

Materials and Methods

Patients and Tissues

Our study design was approved by the medical ethical committee of the University Hospital Rotterdam, the Netherlands.

Nonmalignant Breast Tissues Breast tissue without specific abnormalities from 10 women who underwent reduction mammoplasty was used to study Bcar1/p130Cas levels in normal tissue. Three 50-μm cryosections from these tissues in TS buffer (i.e., 10 mM Tris-HCl [pH 7.5] and 1% sodium dodecyl sulfate [SDS]) were sonicated for 10 seconds and boiled for 10 minutes. Lysates were cleared by centrifugation at 12 000 g for 10 minutes at room temperature, and total protein concentrations were determined with the BCA protein assay (Pierce Chemical Co., Rockford, IL). Bcar1/p130Cas levels were measured with the Western blotting assay described below.

Primary Breast Carcinoma Bcar1/p130Cas levels were determined in cytosol preparations (as described below) from 937 primary breast tumors collected from 1978 through 1988. Selection of samples was based on the availability of stored cytosol extracts (in liquid nitrogen), which remained after routine ER and PgR analyses. Of the 937 patients, 775 (modified mastectomy = 408 patients; breast-

Table 1. **Relationship of Bcar1/p130Cas expression with patient and tumor characteristics at the time of surgery**

Characteristic	Total*	BCAR1/p130Cas expression, %				Two-sided *P*
		None	Low	Intermed	High	
Patients, No.	775	33	39	20	8	
Age, y						.006†
≤40	101	37	41	19	4	
40-55	257	33	44	16	7	
56-70	270	33	35	21	11	
>70	147	27	39	24	10	
Menopausal status						.04‡
Premenopausal	293	34	43	17	6	
Postmenopausal	482	32	37	22	10	
Tumor size§						.86‖
T_1 ≤2 cm	294	32	39	19	9	
T_2 >2-5 cm	372	32	40	20	8	
$T_{3/4}$ >5 cm	97	35	38	20	7	
Lymph node status§						0.81‖
N_0	310	33	39	19	9	
N_{1-3}	224	33	40	20	7	
$N_{>3}$	232	31	41	20	9	
Differentiation grade¶						.01‡
Good/moderate	140	23	45	24	8	
Poor	440	37	38	16	8	
Missing	195	30	38	24	8	
ER status						<.001†
Negative	165	52	30	15	3	
Positive	606	27	42	21	10	
PgR status						<.001†
PgR^-	224	45	33	17	5	
PgR^+	535	27	42	21	10	

*Because of some missing values, numbers do not always add up to 775 patients.
†Wilcoxon-type test for trend.
‡Mann-Whitney test.
§Tumor size and nodal status, see (*29*)
‖Kruskal-Wallis test.
¶Based on histological and cellular characteristics, as stated in the reports of the regional pathologists.

conserving treatment = 367 patients) were eligible for analysis of relapse-free survivors and overall survival according to the criteria described elsewhere (*28*).

Exclusion criteria were patient tissue that was taken from a biopsy only (i.e., inoperable stage T4 tumors or tissue that was not obtained from the primary breast tumor) and patients with insufficient follow-up documentation. Patients who were referred to our institute more than 100 days after primary surgery and patients with distant metastasis at time of primary surgery [M1 patients; staging according to the International Union Against Cancer TNM [tumor-node-metastasis] classification (*29*)] were excluded from analyses of relapse-free survival and overall survival, but they were not necessarily excluded from the analysis of response. In fact, we used 36 such patients in our analysis of the response to tamoxifen therapy in addition to those who were included according to the criteria described below.

Tumor size, lymph node status, and differentiation grade were gathered from the reports of regional pathologists. The differentiation grade is not based on a central pathological review of all tumor samples and, thus, reflects daily practice. The median age of the 775 patients at surgery was 57 years (range, 27-90 years). Radiotherapy was given to 87% of the patients at one or more of the following sites: the breast/thoriacic wall of 549 patients and/or the axilla of 296 patients and/or lymph node areas other than the axilla of 328 patients. Adjuvant chemotherapy (mainly, cyclophosphamide, methotrexate, and 5-fluorouracil [CMF]) was given to 144 patients, and adjuvant hormonal therapy, either alone (38 patients) or in combination with CMF (14 patients), was given to 52 patients. All patients were routinely examined every 3-6 months during the first 5 years of follow-up and once a year thereafter. Of the 775 patients included in the study for analysis of relapse-free survival and overall survival, 442 (57%) patients showed evidence of relapse during follow-up, and 372 (48%) died. At the end of the study, 403 patients were alive, with a median follow-up of 105 months (range, 18-175 months). The following characteristics at surgery are shown in Table 1: tumor size, lymph node status, differentiation grade of the tumor, age, menopausal status, ER status, and PgR status.

Of the 442 patients who relapsed, 232 patients subsequently received tamoxifen therapy as a first-line treatment and were included in the analysis of response to

first-line tamoxifen therapy for recurrent disease. The remaining patients were treated by surgery, radiotherapy, chemotherapy, or other forms of endocrine therapy or died without further treatment. The following inclusion criteria were used: patients with advanced disease who were treated with first-line tamoxifen therapy (40 mg/day) and were not exposed to hormonal treatment at an earlier stage (hormo naive). This subset was expanded to include the 36 patients excluded from analyses of relapse-free survival and overall survival. The median age of the patients with advanced disease at the start of tamoxifen treatment was 63 years (range, 28-91 years). Of the patients, 18% (n = 48) were premenopausal and 82% (n = 220) were postmenopausal. The dominant site of disease was the viscera in 107 patients, the bone in 118 patients, and the soft tissue in 43 patients. After primary surgery, 50 (19%) patients received systemic adjuvant chemotherapy (CMF, 41 patients; 5-fluorouracil, doxorubicin [Adriamycin] and cyclophosphamide [FAC], nine patients). During the course of their metastatic disease, 60% of the patients were subsequently treated with one or more additional hormonal treatments (mostly progestins) after disease progression during the first-line tamoxifen treatment. At the time of analysis, 40 patients were still alive, with a median follow-up of 38 months (range, 5-88 months), and 228 patients had died, with a median survival time of 20 months (range, 11 days to 8 years) after start of tamoxifen therapy. During follow-up but after the start of first-line endocrine therapy, 252 (94%) of 268 patients had disease progression, with a median time to disease progression of 5 months (range, 11 days to 6 years). Patients were examined during endocrine therapy at the outpatient clinic on average once every 6 weeks and during long-term remission up to once every 12 weeks. Response to treatment, as assessed by standard criteria, was defined as a patient having an objective response (complete [i.e., the complete disappearence of all metastases] and partial remission) or prolonged stable disease, with a time to treatment failure of more than 6 months (*30-32*). Patients with prolonged stable disease and patients with a partial remission have similar survival probability (*33,34*). When there was any doubt, the worst category of response was chosen. Of the 268 patients who received tamoxifen therapy as first-line treatment for

recurrent or metastatic disease, 136 (51%) patients responded (42 had an objective response and 94 had stable disease). Of the 132 (49%) patients who did not respond, 17 showed no change in their disease for 6 months or less, and 115 patients showed progressive disease after treatment began.

Preparation of Cytosolic Extracts from Tumors

Cryopreserved (liquid nitrogen) breast tumor specimens were pulverized with a microdismembrator as recommended by the European Organization for Research and Treatment of Cancer (EORTC) for determining cytosolic levels of the ER and PgR proteins (*34*). The resulting tissue powder was suspended in EORTC receptor buffer (10 mM K_2HPO_4, 1.5 mM K_2EDTA, 3 mM sodium azide, 10 mM monothioglycerol, and 10% [vol/vol] glycerol [pH 7.5]). The homogenate was centrifuged at 100 000 x *g* for 30 minutes at 4°C, and the supernatant fraction (cytosol extract) was obtained and stored in liquid nitrogen until assayed. Total protein levels in the extracts were determined using the Bradford dye-binding assay (Bio-Rad Protein Assay; Bio-Rad Laboratories Inc., Hercules, CA) with human serum albumin (Kabi Vitrum Diagnostica, Stockholm, Sweden) as the standard. ER and PgR levels were determined by a ligand binding assay or an enzyme immunoassay as described previously (*36*). The cutoff point used to classify tumors as ER or PgR positive was 10 fmol of receptor protein/mg of cytosol protein.

Western Blot Assay for Bcar1/p130Cas

To determine Bcar1/p130Cas levels in tissue, 5 μg of cytosolic or total extract protein from tumor tissue, nonmalignant breast tissue, or a cell line standard was boiled in loading buffer (i.e., 10 mM Tris-HCl [pH 6.8], 1 mM EDTA, 10% glycerol, 2% SDS, 2.5% 2-mercaptoethanol, and 0.00025% bromophenol blue) and electrophoretically separated in SDS-6% polyacrylamide gel. Each gel also contained prestained SDS-polyacrylamide gel electrophoresis molecular weight markers (Bio-Rad Laboratories) and lysate from the Bcar1/p130Cas-overexpressing human cell line 4A12 (*16*) in two concentrations as a standard.

Proteins were transferred to a poly(vinylidene difluoride) membrane (Hybond P, Amersham Pharmacia Biotech, UK) with a semidry transblotter (Owl, Scientific Inc., Woburn, MA). After an overnight blocking step at 4°C in TBST (i.e., 100 mM Tris-HCl [pH 7.4], 1.5 M NaCl, and 0.05% Tween 20) containing 0.6% bovine serum albumin, the blots were incubated with a 1:5000 dilution of anti-p130Cas monoclonal antibody generated against rat p130Cas (Transduction Laboratories, Lexington, KY) for 1 hour. This procedure was followed by incubation with a 1:10 000 dilution of a horseradish peroxidase-conjugated rabbit anti-mouse antibody (DAKO, Glostrup, Denmark) for 1 hour. Immunocomplexes

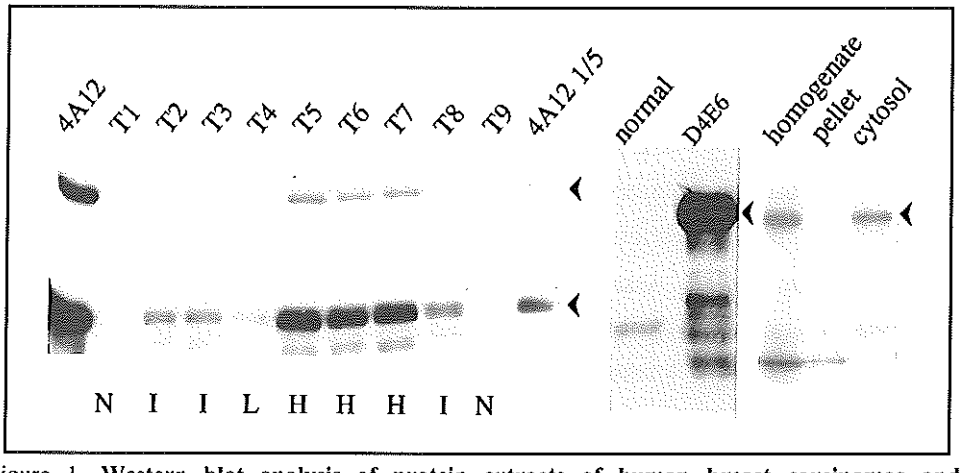

Figure 1. **Western blot analysis of protein extracts of human breast carcinomas and nonmalignant breast tissue.** *Left panel* Five micrograms of cytosol extracts from nine primary breast carcinomas (lanes T1-T9) was examined by Western blot analysis with an anti-p130Cas monoclonal antibody (mAb) to measure Bcar1/p130Cas protein. As a standard, a total lysate from 4A12 cells, a Bcar1/p130Cas-overexpressing human cell line, was applied at two concentrations. Two exposure times of the relevant part of the blot are shown, with the position of the Bcar1/p130Cas band (approximately 116 kd) indicated by an arrowhead. Short (top) and long (bottom) exposures were used to classify Bcar1/p130Cas expression as none (lanes N), low (lanes L), intermediate (lanes I), or high (lanes H). *Middle panel* Five micrograms of a cell lysate from nonmalignant (normal) breast tissue was examined by Western blot analysis with the p130Cas mAb. A very long exposure of the blot is shown. A cell lysate from D4E6 cells, a Bcar1/p130Cas-overexpressing cell line (*16*), was used as control. The lower band visible on the blot resulted from cross-reaction with the secondary antibody. *Right panel* Equivalent amounts of the homogenate and cytosolic (cytosol) and pellet fractions from a primary breast tumor were examined by Western blot analysis with mAb p130Cas.

were visualized by chemiluminescence (enhanced chemiluminescence Western blotting detection reagents; Amersham Pharmacia Biotech) and exposure to blue-light-sensitive x-ray screen film (CEA, Strängnäs, Sweden). To determine Bcar1/p130Cas protein levels, several exposure times (45 seconds, 90 seconds, 3 minutes, and 6 minutes) were used for each blot, and a Bcar1/p130Cas standard (two concentrations) was used as reference. Bcar1/p130Cas protein levels were scored visually by three investigators (S.van der Flier, A.Brinkman and L.C.J. Dorssers) who were not aware of the clinical data. The consensus score was divided into the four following groups: none = no Bcar1/p130Cas protein expression detected on both short and long exposure of the film (e.g., see Fig. 1, lanes T1 and T9); low = no signal on a short exposure and a weak signal on a long exposure (Fig. 1, lane T4); intermediate = a weak signal on a short exposure and a clear signal on a long exposure (Fig. 1, lanes T2, T3, and T8); high = a clear signal on a short exposure (Fig. 1, lanes T5, T6, and T7).

Statistical Analysis

The strength of the association between Bcar1/p130Cas levels in cytosolic extracts from tumors (treated as an ordinal variable) and other prognostic variables (patient or tumor characteristics treated as grouping variables) was tested with the Mann-Whitney U-test or the Kruskal-Wallis test. To test for trends, a nonparametric test for trend across ordered groups as developed by Cuzick (*37*) was used. All *P* values are two-sided. Two-sided *P* values of less than .05 were considered statistically significant. Relapse-free survival and overall survival probabilities were calculated by the actuarial method of Kaplan and Meier (*38*). A local recurrence (n = 442), distant spread (n = 313), or contralateral breast cancer (n = 37) was considered an event for relapse-free survival. For the analysis of relapse-free survival, patients who died without a reported disease recurrence were censored at their date of death. Both univariate and multivariate analysis were performed with the Cox proportional hazard model. For missing values, separate dummy variables were created. The

assumption of proportional hazards was verified graphically. Relative hazard ratios were calculated and presented with 95% confidence intervals. For multivariate analysis, a basic model was introduced, including the classical prognostic factors (age, menopausal status, tumor size, number of positive lymph nodes, ER status, PgR status, differentiation grade, and systemic adjuvant therapy). Age and menopausal status combined were treated as one variable. The prognostic importance of Bcar1/p130Cas expression was tested by adding Bcar1/p130Cas to the basic model. The likelihood ratio test in the univariate Cox regression model was used to test for differences and the log-rank test for trend was used to evaluate trends. The relation with response to therapy was examined with logistic regression analysis. For analysis of the time to disease progression after the start of tamoxifen treatment, the Cox proportional hazard model was used. All statistical analyses were performed with Stata Statistical Software (release 5.0; Stata Corporation, College Station, TX).

Results

Detection of Bcar1/p130Cas Protein by Western Blot Analyses

The anti-p130Cas monoclonal antibody used recognizes the human Bcar1/p130Cas protein with an apparent molecular mass of 116 kd, as estimated by SDS-polyacrylamide gel electrophoresis (*16*). By analyzing subcellular fractions, we retained the bulk of Bcar1/p130Cas protein in routinely prepared cytosolic extracts (Fig. 1), thus enabling the screening of a large number of breast carcinomas for Bcar1/p130Cas expression. As shown by Western blot analysis, levels of Bcar1/p130Cas protein differed in different tumors (Fig. 1). Bcar1/p130Cas protein expression was also evaluated in nonmalignant breast tissue from 10 women. In contrast to malignant tissue, Bcar1/p130Cas protein was not detected in any nonmalignant breast tissue analyzed (Fig. 1). To study the possible relationship with relapse-free survival and overall survival and response to first-line tamoxifen treatment, the expression levels of Bcar1/p130Cas from breast tumors were divided into four groups (none, low, intermediate, and high) based on the intensity of the Western blot bands.

Relationship of Bcar1/p130Cas Levels with Patient and Tumor Characteristics

We compared the levels of Bcar1/p130Cas protein in cytosolic extracts from 775 tumors with patient and tumor characteristics (Table 1). Thirty-three percent of the tumors had no Bcar1/p130Cas protein, 39% had low Bcar1/p130Cas expression, 20% had intermediate expression, and 8% had high Bcar1/p130Cas expression. Intermediate and high levels of Bcar1/p130Cas were more frequently found in tumors from older patients (P=.0063). No statistically significant

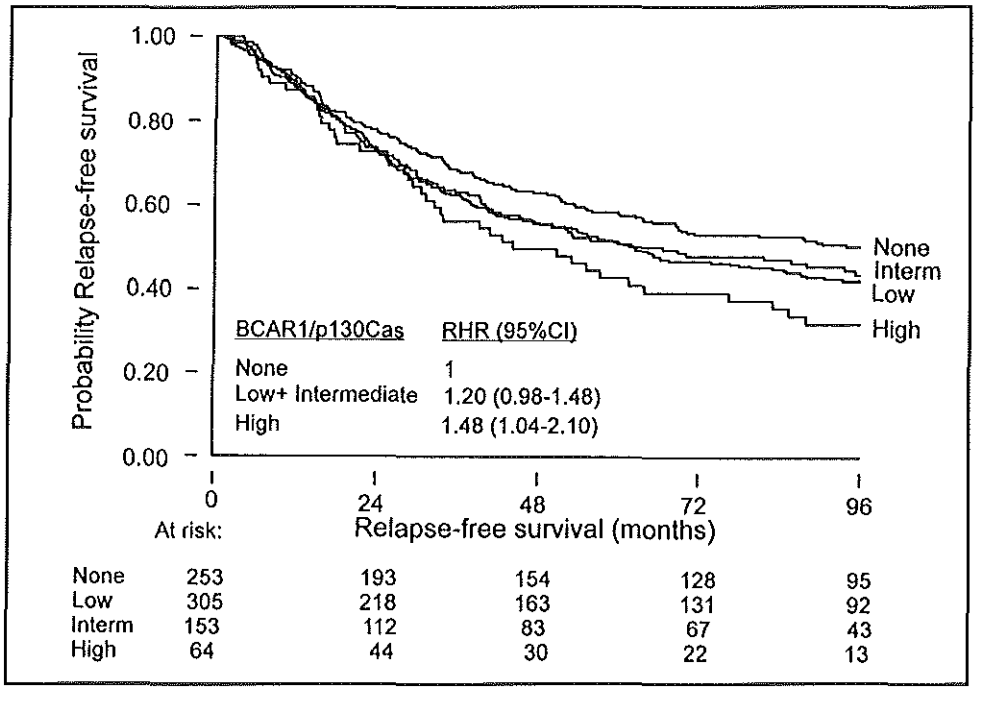

Figure 2. **Relapse-free survival of 775 breast carcinoma patients as a function of the expression level (none, low, intermediate [interm], or high) of Bcar1/p130Cas protein in the cytosolic extracts of primary breast carcinomas.** The number of patients at risk are indicated. The survival probability (and their 95% confidence intervals [CI]) at 48 and 96 months, respectively are as follows: curve none= 0.63 (95% CI = 0.57- 0.69) and 0.50 (95% CI = 0.44- 0.56); curve low= 0.56 (95% CI = 0.50- 0.61) and 0.42 (95% CI= 0.36- 0.48); curve intermediate= 0.56 (95% CI = 0.47- 0.63) and 0.44 (95% CI = 0.35-0.52); curve high=0.50 (95% CI = 0.37- 0.61) and 0.32 (95% CI = 0.20- 0.44). RHR = relative hazard rate.

association of Bcar1/p130Cas levels with tumor size or lymph node status was observed. Reduced levels of Bcar1/p130Cas expression (P = .01) were statistically significantly associated with poorly differentiated tumors. Higher Bcar1/p130Cas levels were statistically significantly associated with ER and PgR positive tumors (P<.001). Expression levels of Bcar1/p130Cas were similar in tumors from patients who did not receive adjuvant treatment or who received adjuvant hormonal therapy or chemotherapy with or without hormonal therapy (χ^2 = 9.79, df = 6; P = .13).

Bcar1/p130Cas and Relapse-Free and Overall Survival

We next studied the relationship between the level of Bcar1/p130Cas protein in primary tumors and the length of relapse-free survival. We found a statistically significant association of high Bcar1/p130Cas levels with poor relapse-free survival (logrank test for trend; χ^2 = 4.38, df = 1; P = .04) and poor overall survival (logrank test for trend; χ^2 = 4.01, df = 1; P = .04). Patients with low and intermediate levels of Bcar1/p130Cas protein in their primary tumor had similar periods of relapse-free survival (Fig. 2). On the basis of this observation, we decided to combine these levels for further analyses. Multivariate analysis was used to evaluate the possible independent prognostic importance of Bcar1/p130Cas expression in analyses of relapse-free survival and overall survival (Table 2). We introduced a basic model that included age, menopausal status, tumor size, number of positive lymph nodes, and the dichotomized biochemical parameters ER and PgR status. When we added the Bcar1/p130Cas level as an ordinal variable to the basic models for relapse-free survival and overall survival, we found that its expression level contributed to the prognostic information provided by the classical prognostic factors (Table 2). Thus, in patients with primary breast cancer, Bcar1/p130Cas is an independent predictor of early recurrence and death.

Bcar1/p130Cas and Response to Tamoxifen Treatment

Next, we investigated a possible relationship between the level of Bcar1/p130Cas

Table 2. **Multivariate Cox regression analysis**

Factor	Relapse-free survival		Overall Survival	
	Two-sided *P*	RHR* (95% CI*)	Two-sided *P*	RHR* (95% CI*)
Basic model†				
Age/menopausal status	.025		.21	
Premenopausal‡		0.76 (0.59-0.99)		0.91 (0.66-1.27)
Postmenopausal‡		0.95 (0.82-1.10)		1.17 (1.01-1.36)
Postmenopausal versus premenopausal		1.09 (0.71-1.68)		1.01 (0.60-1.70)
Tumor size§	.012		.002	
T_2 versus T_1		1.15 (0.92-1.43)		1.36 (1.05-1.75)
$T_{3/4}$ versus T_1		1.68 (1.23-2.29)		1.95 (1.40-2.73)
Missing versus T_1		0.82 (0.33-2.01)		1.59 (0.73-3.47)
Lymph node status§	<.001		<.001	
N_{1-3} versus N_0		2.22 (1.66-2.97)		2.37 (1.72-3.26)
$N_{>3}$ versus N_0		3.36 (2.54-4.44)		3.33 (2.46-4.52)
Missing versus N_0		2.04 (0.82-5.07)		1.83 (0.73-4.62)
ER* status	.038		.036	
Positive versus negative		1.18 (0.89-1.54)		0.99 (0.75-1.31)
Missing versus negative		5.06 (1.70-15.04)		5.77 (1.83-18.2)
PgR* status	.76		.025	
Positive versus negative		0.92 (0.72-1.18)		0.70 (0.54-0.90)
Missing versus negative		1.04 (0.52-2.09)		0.86 (0.43-1.73)
Systemic adjuvant therapy	<.001		.005	
Hormonal therapy versus none		0.64 (0.42-0.99)		0.66 (0.42-1.03)
Chemotherapy ± hormonal therapy versus none		0.50 (0.37-0.69)		0.60 (0.42-0.85)
Differentiation grade¶	.004		.26	
Poor versus good/moderate		1.30 (1.00-1.70)		1.26 (0.94-1.69)
Missing versus good/moderate		0.90 (0.66-1.23)		1.12 (0.80-1.57)
Addition to basic model				
BCAR1/p130Cas#	.03		.04	
L + I versus none		1.20 (0.97-1.50)		1.24 (0.98-1.57)
High versus none		1.62 (1.13-2.32)		1.65 (1.11-2.46)

*RHR = relative hazard rate; CI = confidence interval; ER = estrogen receptor; PgR, progesterone receptor; L = low; I = intermediate.

†Basic model included age/menopausal status (combined), tumor size, nodal status, ER status, PgR status, systemic adjuvant therapy, and differentiation grade.

‡Age in decades tested separately for pre- and postmenopausal patients.

§ Tumor size and nodal status, see (*29*)

‖Chemotherapy with (n=14) and without (n = 144) hormonal (H) therapy.

¶ See Table 1.

#Added to basic model. Final model included 775 patients.

protein in the tumor and the efficacy of response to tamoxifen as first-line systemic treatment for recurrent or metastatic disease. Of the 268 patients who were treated with tamoxifen, 136 (51%) patients responded, with a median time to progression of 16 months. The responses to tamoxifen were similar in the subgroups with no (53%), low (50%), or intermediate (58%) levels of Bcar1/p130Cas protein in their tumors, whereas the response in patients with a high level of Bcar1/p130Cas in their tumors was lower (33%). However, the objective response was similar in all subgroups. The observed lower response rate in the high Bcar1/p130Cas subgroup is thus a reflection of a reduced number of patients with long-term stable disease. The tumors with high Bcar1/p130Cas levels were predominantly ER-positive (29 [97%] of 30 tumors); fewer patients in the other subgroups had ER-positive tumors (approximately 85%). Again, the subgroups with low and intermediate Bcar1/p130Cas expression were combined. When analysis was performed on 230 ER-positive tumors only, there was a statistically significant association between a poor response to tamoxifen treatment and high expression of Bcar1/p130Cas (high = 34%; none = 61%; P = .02). In the multivariate analysis for response to treatment, Bcar1/p130Cas levels were corrected for menopausal status, relapse-free interval, and ER status (as a dichotomized variable). Independent predictors of poor response were premenopausal status, short disease-free interval, ER-negative status of the tumor, and high levels of Bcar1/p130Cas (Table 3). In a separate analysis, we included ER status as a continuous variable (log transformed) and found that a high level of Bcar1/p130Cas expression, compared to no expression, was still associated with a poor response to tamoxifen (P = .01). However, these results should be interpreted with some caution because we did not correct for multiple comparisons. An explanation for the observed differences between univariate and multivariate analyses could be the strong relation between an ER-positive status of the tumors and high Bcar1/p130Cas expression, which was corrected in the multivariate analysis.

The association between Bcar1/p130Cas expression and time to progression of the disease after start of systemic treatment with tamoxifen, as analyzed by Cox

regression analysis, was not statistically significant. Nevertheless, with increasing expression levels of Bcar1/p130Cas in the primary tumor, the median progression-free survival time of the patients was reduced substantially from 7 months (none, low, and intermediate expression) to 3 months (high expression).

Discussion

An understanding of the molecular mechanisms of tamoxifen resistance should contribute to the determination of optimal therapies for individual patients. We have proposed that genetic alterations in the tumor cells allow these cells to escape antiestrogen therapy (*13*). With our model system, we have identified the BCAR1 gene and shown that it can confer tamoxifen-resistant cell proliferation when overexpressed *in vitro* (*16*). In this study, we investigated a possible association of the expression of Bcar1/p130Cas protein in primary human breast carcinoma with disease progression and response to tamoxifen therapy. Bcar1/p130Cas expression was found to vary considerably between the breast carcinomas, and relatively high Bcar1/p130Cas expression was detected in 8% of the breast primary tumors tested. In contrast, Bcar1/p130Cas protein was not detected in nonmalignant breast tissue samples by western blot analysis. Using the same antibody, immunohistochemical analysis has confirmed that the Bcar1/p130Cas protein detected in cytosolic extracts of tumors by Western blot analysis is localized in tumor cells and that the expression of Bcar1/p130Cas in normal breast tissue is observed in the limited numbers of ductal epithelial cells and not in the abundant stroma (van der Flier S., van der Kwast T.H., and Dorssers L.C.J., unpublished results).

Analysis of the expression of Bcar1/p130Cas protein in primary breast cancer has led to two distinct observations. First, relatively high expression of Bcar1/p130Cas in primary breast tumors is associated with a rapid recurrence of the disease. The risk of relapse was 62% greater for patients whose tumors had high levels of Bcar1/p130Cas than for patients whose tumors had undetectable levels of Bcar1/p130Cas protein. Multivariate analysis showed that a high level of Bcar1/p130Cas in the primary tumor was associated with an early relapse,

Table 3. **Logistic regression analysis of response to tamoxifen therapy**

	No. of Patients	Univariate analysis %†	Univariate analysis Two-sided *P*	Multivariate analysis Odds ratio	Multivariate analysis 95% CI *	Multivariate analysis Two-sided *P*
All patients	268	51				
Menopausal status						
Premenopausal	48	44		1		
Postmenopausal	220	53	.02	2.54	1.26-5.09	.01
Dominant site of relapse						
Soft tissue	43	53				
Bone	118	51	.77	—		
Viscera	107	50	.66			
Relapse-free survival, mo						
< 12 months	74	30		1		
≥ 12 months	194	59	<.001	3.29	1.79-6.06	<.001
ER status						
Negative	37	22		1		
Positive	230	56	<.001	4.47	1.89-10.59	<.001
PgR status						
Negative	64	36				
Positive	196	56	.01	—		
BCAR1/p130Cas level‡						
None	72	53		1		
L+I versus none	166	53	.97	0.90	0.49-1.66	.74
High versus none	30	33	.08	0.38	0.14-0.98	.04

*CI = confidence interval; ER = estrogen receptor; PgR = progesterone receptor.L=low; I=intermediate. There was one missing value for ER and eight for PgR.

†Percent overall response = percentage of patients with complete response, partial response, or stable disease for more than 6 months.

‡ Distributions of BCAR1/p130Cas expression in tumors of the group of 36 patients included were not different from the remaining 232 patients selected from the disease-free survival analysis (Table 1) [$\chi 2 = 2.80$, $df = 3$, $P = .42$].

independent of the classical prognostic parameters. Consequently, a high level of Bcar1/p130Cas protein in primary tumor extracts (compared with no detectable Bcar1/p130Cas in their primary tumor) is statistically significantly associated with a 65% reduced overall survival of patients. These results indicate that tumors with high-level expression behave more aggressively than tumors with low-level

or undetectable expression. This association may be explained biologically by the involvement of Bcar1/p130Cas in cellular migration and invasion (*21-23*). These properties suggest a higher risk of early dissemination of individual cells from the subgroup of tumors with high-level expression of Bcar1/p130Cas.

Second, high-level expression of Bcar1/p130Cas in the primary tumors (compared with tumors with no detectable Bcar1/p130Cas expression) is associated with a poorer response to first-line tamoxifen treatment. This observation is consistent with our findings with the *in vitro* model system; i.e., Bcar1/p130Cas overexpression resulted in antiestrogen resistance in cell lines (*13,16*). Tamoxifen generally induces an objective response or disease stabilization in about half of the patients with ER-positive primary tumors and recurrent or metastatic disease (*39,40*). The other patients who had ER-positive primary tumors experience immediate disease progression due to intrinsic resistance to tamoxifen. In our study, 67% of patients with a relatively high level of Bcar1/p130Cas protein in their primary tumors did not respond to tamoxifen. This finding may explain some of the clinically observed intrinsic resistance to tamoxifen. Because these tumors are generally ER positive and the patients are, thus, eligible for tamoxifen therapy, Bcar1/p130Cas levels could be used by physicians to determine which patients may not respond to tamoxifen. Therapeutic decisions may benefit from more precise quantification of Bcar1/p130Cas protein levels in primary tumors. Because several lines of evidence show that development of resistance to antiestrogen agents is not always associated with the loss of ER expression in the tumor cells (*41-43*), the association of the high levels of Bcar1/p130Cas in ER-positive primary tumors that have a poor response to tamoxifen suggests the possibility of an alternative pathway to bypass estrogen-receptor-mediated cell proliferation.

Despite many papers on Bcar1/p130Cas function in various cell systems, few clues are available about the pathway leading to the altered proliferation control in our cells (*44,45*). Changes in adhesion and migration may affect cell proliferation, but the precise pathway in breast epithelial cells is yet unclear. Bcar1/p130Cas protein can be phosphorylated on tyrosine residues and can

associate with many proteins that contain SH2 domains (*44,46,47*). In addition, the SH3 domain of Bcar1/p130Cas may also associate with other proteins through proline-containing target sequences (*17,48,49*). Bcar1/p130Cas serves as an adapter protein to contact proteins that participate in various cellular processes and is dependent on the availability, activation status, and affinity of its partners. These interactions are part of a cascade of signaling events leading to the regulation of gene expression and regulation of the cell cycle. Because Bcar1/p130Cas appears to have a role in the progression of clinical breast cancer, elucidation of this critical pathway could contribute valuable information for the development of new treatment strategies for antiestrogen-resistant breast cancer.

Notes

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2.3

Comparison of BCAR1/p130Cas in untreated and acquired tamoxifen-resistant breast carcinomas

Chapter 2.3

Comparison of BCAR1/p130Cas Expression in Untreated and Acquired Tamoxifen-resistant Human Breast Carcinomas

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Abstract

High BCAR1/p130Cas expression, as determined in primary breast tumor cytosols, predicts a poor chance of response of recurrent or metastatic disease to tamoxifen treatment in patients with estrogen receptor (ER)-positive breast carcinomas. However, changes in BCAR1/p130Cas expression on acquisition of resistance have not been investigated. The aim of this study was to assess whether BCAR1/p130Cas expression is altered during acquisition of anti-estrogen resistance. By chemiluminescent Western blot analysis BCAR1/p130Cas protein was quantitatively measured in cytosols of a panel of 34 ER-positive carcinomas which initially responded to primary tamoxifen treatment and subsequently progressed (n=22) or which developed during adjuvant tamoxifen treatment (n=12). These tamoxifen-resistant carcinomas were compared to a panel of 54 untreated ER-positive human breast carcinomas. A BCAR1/p130Cas-specific antibody was used in immunoblotting to compare the samples side by side with a dilution series of protein standard for BCAR1/p130Cas. In this analysis we did not detect significant differences in the level of BCAR1/p130Cas expression between untreated and acquired tamoxifen-resistant carcinomas. These results indicate that in tumor progression towards tamoxifen-resistance, increase in expression of BCAR1/p130Cas is perhaps only one of the possible molecular mechanisms. A high BCAR1/p130Cas protein level appears a hallmark of the breast carcinomas for intrinsic resistance to tamoxifen.

Introduction

The anti-estrogen tamoxifen is used as a systemic hormonal treatment of pre- and postmenopausal women with all stages of breast cancer. Tamoxifen produces significant increases in both disease-free and overall survival (*1,2*) and is palliative to patients with advanced disease. Clinical outcomes of breast cancer are related to ER expression because ER-positivity of tumors increases the probability that an individual breast cancer patient will respond to anti-estrogen therapy (*3*). Age of the patient is not a determining factor in the feasibility of response to tamoxifen treatment, since premenopausal women benefit from

tamoxifen as much as postmenopausal women (*2,4*). About half of the patients with ER-positive tumors show immediate disease progression due to intrinsic resistance to tamoxifen. Unfortunately, the majority of initially tamoxifen-responsive, ER-positive tumors also eventually becomes resistant to tamoxifen therapy (acquired resistance), resulting in disease progression. In most cases, the tumor cells continue to express ER but are no longer responsive to tamoxifen (*5,6*). Several studies indicate that tamoxifen resistance may be multifactorial (*7,8*). We have hypothesized that (epi-)genetic alterations within the tumor cells allow the cells to escape the anti-estrogen therapy (*9,10*). Dysregulation and overexpession of genes that control cell proliferation may contribute to tumor progression on tamoxifen therapy. Previously, we have identified the breast cancer anti-estrogen resistance gene (BCAR1/p130Cas) using an *in vitro* model system. Furthermore we have shown that overexpression of this gene *in vitro* suffices to convert breast cancer cells that are growth inhibited by anti-estrogens into anti-estrogen-resistant proliferating cells (*11*). The BCAR1/p130Cas protein is an adapter protein with multiple phosphorylation sites and a Src Homology 3 (SH3)-domain which serves as a multifunctional signal assembly in an interacting signaling network contributing to the signaling control of cellular processes as spreading, adhesion (*12-14*), and cell invasion and migration (*15,16*).

In an earlier study we have demonstrated that ER-positive primary breast carcinomas with relatively high expression of the BCAR1/p130Cas protein are more likely to recur suggesting an increased metastatic potential. Moreover, the recurrences of the primary carcinomas with high BCAR1/p130Cas expression are predicted to be poor responders to first-line tamoxifen treatment (*17*). This latter finding provides clinical evidence to support a role for high expression of BCAR1/p130Cas protein in intrinsic resistance to tamoxifen treatment of ER-positive breast carcinomas. In the present study, we have attempted to determine if the BCAR1/p130Cas protein may have a role in the acquisition of tamoxifen resistance and subsequent breast cancer progression. To monitor possible changes in BCAR1/p130Cas protein expression, which may reflect acquisition of tamoxifen resistance, we have quantitatively measured BCAR1/p130Cas levels in

primary breast carcinomas and compared these to the BCAR1/p130Cas levels in carcinomas that progressed after acquiring resistance to tamoxifen.

Materials and Methods

Human carcinomas

Two categories of breast carcinomas with tamoxifen resistance were selected from a previously reported sample collection at the Royal Marsden Hospital (*18*): (a) the acquired resistance category consisted of 22 primary breast tumors from patients with a median age of 74 years (range 36-86 years) at the time of surgery after receiving tamoxifen as primary medical therapy and becoming resistant: these patients had initially shown an objective clinical response to tamoxifen treatment; (b) the adjuvant resistance category consisted of locally relapsed tumors from 12 patients with a median age of 62 years (range 28-94 years) at the time of surgery: resistance developed after ≥ 2 years on adjuvant tamoxifen treatment. All patients had continued their tamoxifen treatment untill the time of surgery. Both of these panels consisted of ER-positive tumors except for two cases (Table 1). Patients that exhibited intrinsic resistance to tamoxifen treatment were excluded from the treatment group. The untreated group was also selected from the tumor bank at the Royal Marsden Hospital and consisted of 54 untreated, ER-positive, primary carcinomas (n=54) from patients with a median age of 67.5 years (range 51-83 years) at the time of surgery. The only criteria other than positive ER status (to match the treatment group) for selection of this control group was sufficient mass (100-200 mg) of stored tissue for analysis. After excision, the tissue samples were cryopreserved (-80°C).

Cytosolic extracts from carcinomas

Frozen breast tumor tissues (100-200 mg) were pulverized in a tissue dismembrator (Braun Biotech, Buckinghamshire, UK) for one minute. The resulting frozen tissue powder was homogenized in 500 µl iced extraction buffer containing several protease and phosphatase inhibitors (50 mM Hepes pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 1% Triton-X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 200 µM Na orthovanadate, 10 mM

pyrophosphate, 100 mM NaF, 100 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1μg/ml aprotinin). Cytosols (supernant fractions) were prepared by centrifugation of the tumor homogenates at 4°C for 5 min at 10,000g and

Table 1. **Patient and tumor characteristics at time of surgery**

Variable	No. of patients	No. of untreated patients	No. of patients with primary tamoxifen treatment	No. of patients with adjuvant tamoxifen treatment
All patients	88	54	22	12
Menopausal status[a]				
Premenopausal	5	-	2	3
Postmenopausal	83	54	20	9
ER status of tumor[b]				
Negative	2	-	1	1
Positive	86	54	21	11

[a] menopausal status: patients ≤50 years, postmenopausal status: patients >50 years; [b] cut-off point to classify tumors as estrogen receptor-positive and –negative was 10 fmol of receptor protein per mg of cytosol protein

stored at –80°C until assayed. Total protein concentrations were determined using the Bradford dye-binding assay (Bio-Rad Protein Assay, Bio-Rad Laboratories, Hercules, CA). Total immunoreactive ER was assayed in the cytosol by ER EIA (Abbott Laboratories, Illinois, IL).

Western blot procedure for BCAR1/p130Cas

One quarter of a μg of cytosolic extract was electrophoresed on a 6% sodium dodecyl sulfate-polyacrylamide gel. Each gel contained molecular weight markers and, as a standard four serial dilutions (from 1 to 0.15 μg) of lysate from the BCAR1/p130Cas-overexpressing human cell line 4A12 (*11*). After transfer of the proteins onto nitrocellulose membranes (Schleicher & Schuell Nederland BV, 's

Hertogenbosch, the Netherlands), filters were blocked in 2% milkpowder. Incubation was performed with αp130Cas, the primary monoclonal antibody against rat (Transduction Laboratories, Lexington, KY), diluted 1 to 5000 in TBS-Tween (10 mM Tris pH 7.4, 150 mM NaCl and 0.025% Tween 20) containing 1.5% BSA. After PBS-Tween washing, the filters were incubated with horseradish peroxidase conjugated sheep anti-mouse antibody (Amersham Pharmacia Biotech, Rainham, UK) diluted 1 to 2000 containing 1% milkpowder. After several washes, immunoreactive BCAR1/p130Cas protein was visualized by chemiluminescence (Supersignal Ultra-Chemiluminescent Substrate; Pierce, Rockford, IL) on blue sensitive X-ray film (Fuji, Tokyo, Japan).

Digital image collection and analysis

A cooled CCD-based imaging system has been applied to image the chemiluminescent Western blots and to quantitate the BCAR1/p130Cas band intensities on the blots (Fluor-S MultiImager: CCD cooled camera, 1340x1037 pixels, 256 gray levels, dynamic range of 12 bits; aspherical lens SP AF 20-40 mm, F/2.7-3.5; Bio-Rad Laboratories). For each blot a short (5 minutes) and long (8 minutes) scan time was used to acquire the images. The Multi-Analyst

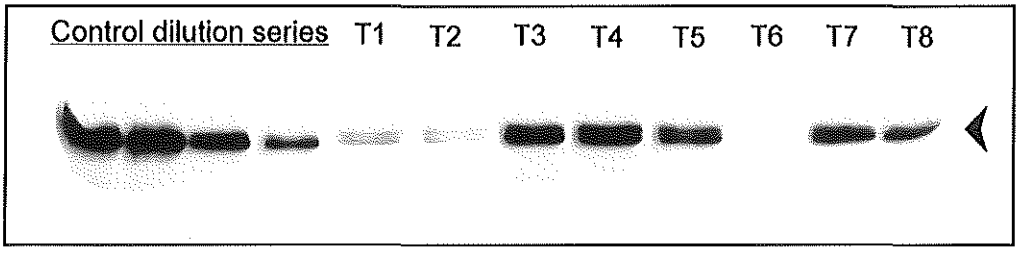

Figure 1. **Western blot of protein extracts of human breast carcinomas.** One quarter of a microgram of cytosolic extract from six untreated primary breast carcinomas (lanes T1-T6) and two tamoxifen-resistant breast carcinomas (lanes T7 and T8) was analyzed by quantitative measurement of chemiluminescent Western blot signals, that were detected using the anti-p130Cas monoclonal antibody. As standard, a dilution series of total lysate from 4A12 cells, a BCAR1/p130Cas-overexpressing human cell line, was applied at four concentrations (1μg, 0.67μg, 0.33μg and 0.15μg). The relevant part of the blot is shown with the position of the BCAR1/p130Cas band (approximately 116 kDa) indicated by an arrowhead.

Software version 1.1 (Bio-Rad Laboratories) was used for automatic analysis of the images. The volume intensity of the BCAR1/p130Cas specific signals was calculated as the intregrated intensity of all pixels within the selected box corrected for the global background. The global background was determined by the mean pixel intensity of several (n=6) defined boxes at representative sites at the digitized blot and subtracted from the intensity of the specific signals resulting in the adjusted volume. Series of pixel intensities were verified to lie within a linear dynamic range. For each blot a standard curve was determined by plotting the arbitrarily units of BCAR1/p130Cas protein determined for the serial dilutions of the BCAR1/p130Cas standard against the integrated volume of the imaged BCAR1/p130Cas bands. The units of BCAR1/p130Cas protein in the tumor cytosols were calculated from these standard curves.

Statistics

The median scores of the BCAR1/p130Cas units of protein for the 3 clinical subgroups (untreated, acquired and adjuvant) was compared by using the Kruskal-Wallis test using STATA statistical software (Stata Corporation, College Station, TX, USA). Two-sided P-values < 0.05 were considered as statistically significant. Box-Whisker plots were used to depict the units of BCAR1/p130Cas protein.

Results

Of the 34 patients in the treated group, 29 were postmenopausal and 5 were premenopausal whereas all patients in the untreated group were postmenopausal (Table 1). The group of untreated tumors was selected for ER-positivity, since the majority of the treated tumors were also ER-positive. As previously reported, the human BCAR1/p130Cas protein is immunoreactive with the αp130Cas antibody used and is detected as a protein with an apparently molecular weight of 116 kDa under denaturing conditions on the immunoblots (Figure 1) (*11*). Double bands for the BCAR1/p130Cas protein were visible on the immunoblots with the applied protocol (Figure 1), most probably indicating the differential levels of

phosphorylation of the BCAR1/p130Cas protein (*19*). For each tumor the intensity of both BCAR1/p130Cas bands together was determined in the quantitative analysis procedure.

Arbitrary units of BCAR1/p130Cas protein were determined for each tumor cytosol for the short as well as the long scan time. When these short and long

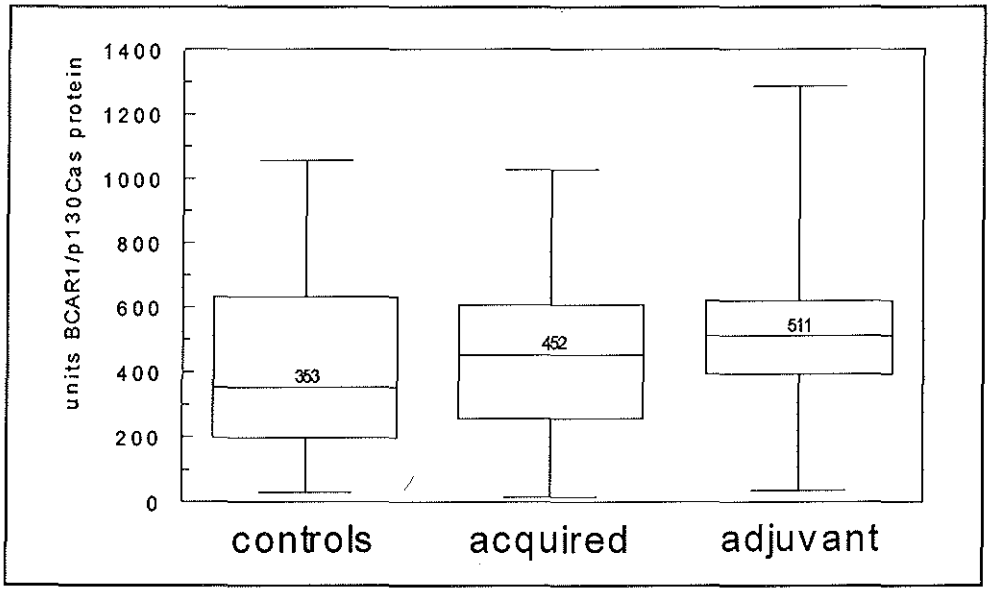

Figure 2. **BCAR1/p130Cas protein in untreated and tamoxifen resistant breast carcinomas.** Box-Whisker graph comparing the units of BCAR1/p130Cas protein in tumor cytosols from untreated control patients (n=54) and patients with acquired tamoxifen resistance primary treated with tamoxifen (n=22) or adjuvant treated with tamoxifen (n=12). Boxes show the range between the 25th and 75th percentiles, with the horizontal lines and the numbers indicating the medians for the data (P=0.59, Kruskal-Wallis Test). The whiskers extend to the 5th and 95th percentiles.

values were plotted against each other a proportionate relation was found. The values obtained by the long exposure were used in the analysis. The levels of BCAR1/p130Cas varied widely between tumors in each group of patients.

The median scores of units of BCAR1/p130Cas protein in the acquired (median 452, range 3-1118) and adjuvant group (median 511, range 35-843) were higher than in the untreated group (353, range 12-1555), but there was no statistically

significant difference in median units of BCAR1/p130Cas protein between the groups (Figure 2).

Discussion

Acquired tamoxifen resistance restricts the benefits of tamoxifen treatment in many breast cancer patients. To identify possible mechanisms for the emergence of tamoxifen-resistant cell growth, examination of variant or mutant estrogen receptors and changes in estrogen receptor DNA binding has been performed (*5,18,20-24*), but so far no predominant mechanism has emerged.

Patients exhibiting tumor progression while on tamoxifen often continue to express the ER (*25*), suggesting that growth-promoting mechanisms independent of the estrogen regulatory pathway are acting. On the other hand, loss of ER expression in recurrent breast cancer may be considered as a result of hormone-independence (*6*) due to an alternative proliferation pathway functioning in these cells.

Recently we have demonstrated that in primary breast carcinomas, a higher level of BCAR1/p130Cas was found in parallel with an ER and PgR positive status of the tumors (*17*). Moreover, the subgroup of primary carcinomas with a high BCAR1/p130Cas level exhibited a worse response to tamoxifen treatment (*17*). Immunohistochemical analysis of breast carcinomas has confirmed that the majority of the BCAR1/p130Cas protein as determined in the tumor cytosols, is present in the carcinoma cells and the vessels of the tumor tissue (*26*).

In this study we have attempted to examine if overexpression of BCAR1/p130Cas is a mechanism involved in the acquisition of tamoxifen resistance *in vivo*. Using quantitative analysis of chemiluminescent Western blot signals, we have now demonstrated that expression of BCAR1/p130Cas protein is not significantly increased in ER-positive breast carcinomas with acquired tamoxifen resistance as compared to untreated carcinomas with a defined risk of being non-responsive to tamoxifen treatment. Considering this absence of significant changes in BCAR1/p130Cas protein between the different groups observed in this study, there is no indication to presume that BCAR1/p130Cas is actively involved in

acquired resistance to tamoxifen and tumor progression in the majority of the cases. However, the current study does not exclude the possibility that increased BCAR1/p130Cas expression could contribute to acquired tamoxifen resistance in a subgroup of patients. To determine this, evaluation of a much extended series of ER-positive untreated and acquired tamoxifen resistant carcinomas would be required. However, clinical materials to conduct such a study are not readily available, particularly if the reagents require the use of frozen tissues. A detailed examination of matched sets of tumor samples pre- and post-tamoxifen treatment could also provide valuable data about the role of BCAR1/p130Cas in development of tamoxifen resistance but again the material to accomplish this is not readily available. Immunohistochemical analysis of BCAR1/p130Cas expression on paraffin-embedded sections would make such a study more viable.

For many of the breast carcinomas studied, multiple bands were detected on the Western blots, suggesting that different phosphorylated forms of the BCAR1/p130Cas protein are present in the breast carcinoma cells/tissues. Most functions accredited to the BCAR1/p130Cas protein - like cell transformation, spreading, adhesion, migration, and invasion - have been shown to be tyrosine-phosphorylation dependent (*27,28*) and tyrosine-phosphorylation of the BCAR1/p130Cas protein has been observed in response to several factors (*12,29-31*). In addition to measuring BCAR1/p130Cas protein levels, future research focussing on the elucidation of the specific phosphorylation patterns could help resolve the role of the BCAR1/p130Cas protein in the growth-promoting pathway in the breast (carcinoma) cells.

In conclusion, the results of this study combined with our previous findings led us to conclude that high BCAR1/p130Cas expression in breast carcinoma cells is associated with intrinsic but not significantly with acquired resistance to tamoxifen.

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2.4

An immunohistochemical study of the BCAR1/p130Cas protein in non-malignant and malignant human breast tissue

Chapter 2.4

An Immunohistochemical Study of the BCAR1/p130Cas Protein in Non-malignant and Malignant Human Breast Tissue

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Abstract

BCAR1/p130Cas is a docking protein involved in intracellular signaling pathways and *in vitro* resistance of estrogen-dependent breast cancer cells to anti-estrogens. The BCAR1/p130Cas protein level in primary breast cancer cytosols was found to correlate with a rapid recurrence of disease in the patients. A high BCAR1/p130Cas level was also associated with a higher chance of resistance to first-line tamoxifen treatment. Using antibodies raised against the rat p130Cas protein, we determined by immunohistochemical methods the BCAR1/p130Cas localization in primary breast carcinomas, in tumors of stromal origin, and in non-neoplastic breast tissues. The BCAR1/p130Cas protein was detected in the cytoplasm of the non-malignant and neoplastic epithelial cells and in the vascular compartment of all tissue sections analyzed. Immunohistochemistry demonstrated a variable intensity of BCAR1/p130Cas staining and a variation in the proportion of BCAR1/p130Cas positive epithelial tumor cells for the different breast carcinomas. The stromal cells in non-malignant tissues and tumor tissues did not stain for BCAR1/p130Cas. This study demonstrates a variable expression of BCAR1/p130Cas in malignant and non-malignant breast epithelial cells.

Introduction

Overexpression of the breast cancer anti-estrogen resistance gene BCAR1/p130Cas has been shown to make estrogen dependent breast cancer cells, anti-estrogen resistant (*1*). BCAR1/p130Cas is the human homologue of the rat p130Crk-associated substrate (p130Cas). The p130Cas protein is a docking protein which in concert with other signaling proteins, is implicated in a variety of biological processes like integrin-mediated cell adhesion (*2-4*), cell survival (*5*), cell migration and invasion (*6-9*), cytoskeletal organization (*10*), growth factor-mediated signal transduction (*11,12*), cardiovascular development (*13*), and bacterial infection (*14,15*).

Previously we have determined BCAR1/p130Cas protein levels in tumor cytosols of primary breast carcinomas by Western blot analysis (*16*). The BCAR1/p130Cas levels in cytosolic extracts of the tissues were found to vary

extensively between the tumors. Relatively high BCAR1/p130Cas expression in primary breast carcinomas appeared to be an independent predictor of a shorter relapse-free survival of the patient and a worse reponse of tumor recurrences to first-line tamoxifen therapy. These results indicate that high BCAR1/p130Cas expression may contribute to the progression of malignant breast tumors.

The molecular pathways responsible for anti-estrogen resistant breast cancer cell proliferation, in which the BCAR1/p130Cas protein is involved, are yet unknown. Northern blot analysis has shown that the BCAR1/p130Cas mRNA is widely expressed in many tissue types (*1*). The BCAR1/p130Cas expression and localization pattern in mammary tissue has as yet not been systematically described. In this study we have immunohistochemically analyzed the BCAR1/p130Cas expression in a series of human breast carcinomas of different histology. We also examined immunohistochemically the BCAR1/p130Cas expression in normal breast tissues, in fibroadenoma and in cystosarcoma phyllodes.

Materials and Methods

Human Breast Tissue Samples

Normal breast tissue and tumor samples were collected and stored in the human tissue bank at the Daniel den Hoed Cancer Center. Randomly selected samples were obtained from 18 primary untreated breast carcinomas and comprised 11 carcinomas of the ductal type and 3 carcinomas of the lobular type, three other samples were carcinomas of a mixed ductal and lobular type and one sample was a papillary carcinoma; 5 breast tissues without specific abnormalities; 5 phyllodes cystosarcomas (graded as benign according to histopathological criteria); a single fibroadenoma and a single myofibroblastoma. The histopathological classification was based on the WHO histopathological typing of breast tumors (*17*). Tissue samples were immediately frozen after excision and stored in liquid nitrogen.

Antibodies

The mouse monoclonal antibody to rat p130Cas (isotype IgG1) was raised against amino acid (aa) residue 644 to 819 (Transduction Laboratories, Lexington, KY, USA) and will be referred to as αp130Cas TL. The mouse monoclonal antibody 8G4-E8 (isotype IgG2a) raised against rat p130Cas aa residue 670 to 896 was a kind gift of Dr. Amy Bouton and Dr. Thomas Parsons, Charlottesville, VA, USA (*18*). Previous immunoblotting experiments demonstrated that both antibodies specifically detect a 116 kDa protein, representative for BCAR1/p130Cas (*1*).

Immunohistochemical staining and analysis

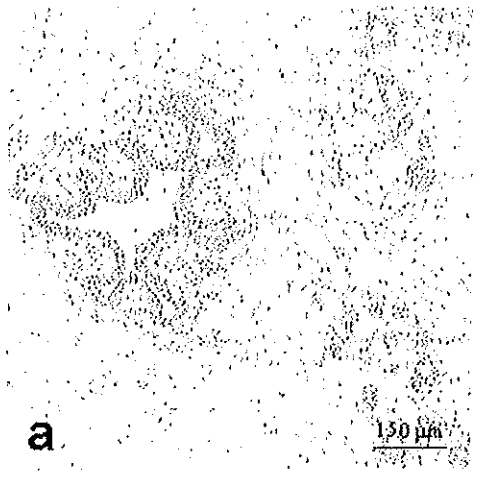
Serial cryosections of 5 μm were placed on clean glass-slides and for each tissue a section was stained with hematoxylin for histological evaluation. The sections for immunohistochemistry were fixed in 4% formaldehyde-phosphate-buffered saline (PBS) for 10 min and then transferred to PBS. After preincubation for 15 min with 5% bovine serum albumine (BSA) in PBS, the sections were incubated with 1 to 2000 diluted α-p130Cas TL antibody for 1 h. After washing in PBS, the slides were incubated with rabbit anti-mouse Immunoglobulins (Dako A/S, Copenhagen, Denmark) diluted 1 to 20 in PBS containing 5% normal human serum (NHS/PBS) for 45 min. After PBS washing the slides were incubated with mouse monoclonal APAAP complex (alkaline phosphatase anti-alkaline phosphatase; Serotec Ltd, Oxford, UK), 1 to 50 diluted in 5% NHS/PBS for 30 min followed by PBS washing. Phosphatase activity was developed with the chromogen Fast Blue/Napthol for 30 min. Finally, the sections were counterstained with Mayer haematoxylin for 1 min. Control experiments consisted of serial tissue sections in which preimmune mouse IgG1 (Dako A/S, Glostrup, Denmark) was used as primary antibody in the staining procedure. Immunohistochemical evaluation was performed by two pathologists (T.H.v.d.K. and S.H-L.) and two other observers (C.J.C.C. and S.v.d.F.). Because the blood vessels always showed a strong immunohistochemical reactivity to the BCAR1/p130Cas antibody in all tissues analyzed, the positively staining blood vessels served as internal control. The staining results for the carcinomas were

recorded as the percentage of BCAR1/p130Cass positive carcinoma cells. The intensity of the staining was recorded separately. Unaffected non-malignant tissue present adjacent to the tumor tissue was assessed for its staining and intensity of BCAR1/p130Cas when present. For comparison with the staining pattern obtained with the αp130Cas TL antibody, three carcinomas were also immunostained with the 8G4-E8 antibody independently raised against the carboxy terminus of rat p130Cas (*19*) using the same protocol.

Western blot assay for BCAR1/p130Cas

The immunohistochemically analyzed carcinomas were also examined for BCAR1/p130Cas expression by Western blot analysis. Tissue lysates were prepared from three 50 μm-cryosections of the carcinoma tissues that were cut consecutively to the sections for the immunohistochemical detection. The tissue lysate preparation, the Western blotting, and the measurement of the BCAR1/p130Cas protein levels in the carcinoma-lysates was performed as described before (*16*). Visual scoring of the Western blot signals for BCAR1/p130Cas was performed based on the intensity of the bands (see Figure 2) according to the three following groups: 0= no BCAR1/p130Cas protein expression ; 1= low BCAR1/p130Cas expression; 2= intermediate BCAR1/p130Cas expression; 3= high BCAR1/p130Cas expression (see also Table 2).

Figure 1. **Immunohistochemical study of BCAR1/p130Cas expression of non-malignant breast tissue (*a*, *b*), breast carcinoma tissue (*c*, *d*), and stromal-derived tumor tissue (*e*, *f*).** Immunostain for BCAR1/p130Cas appears in red, nuclei were counterstained with hematoxylin and appear in blue. Examination of the staining pattern shows a strong immunoreactivity for BCAR1/p130Cas in the blood vessels. In non-malignant human breast tissue the BCAR1/p130Cas positivity was detected in the epithelial cells of the ducts (*a*). At higher magnification (*b*) it becomes evident that the myoepithelial cells lining the ducts stain stronger than the luminal cells. The stromal cells are negative for BCAR1/p130Cas. Expression of BCAR1/p130Cas in carcinoma cells is detected in a ductal carcinoma NOS (*c*) and in an intraductal carcinoma comedo (*d*). The BCAR1/p130Cas antigen is visualized in the epithelial cells of a fibroadenoma (*e*) and of a phyllodes tumor (*f*). In the hypercellular stroma no BCAR1/p130Cas expression was demonstrated.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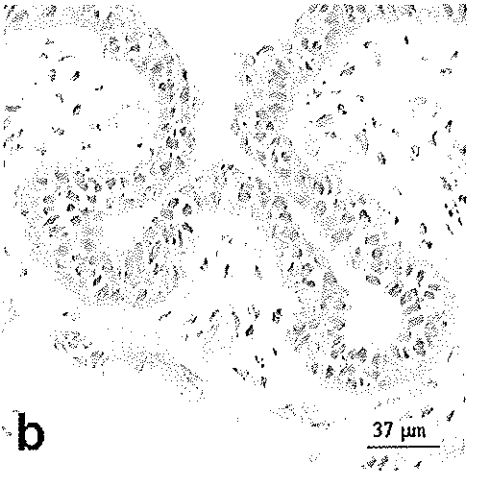
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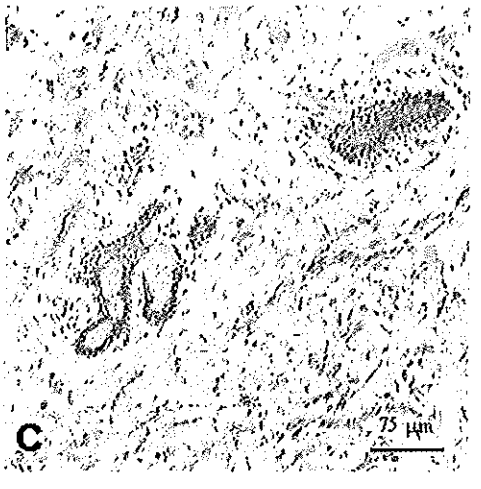
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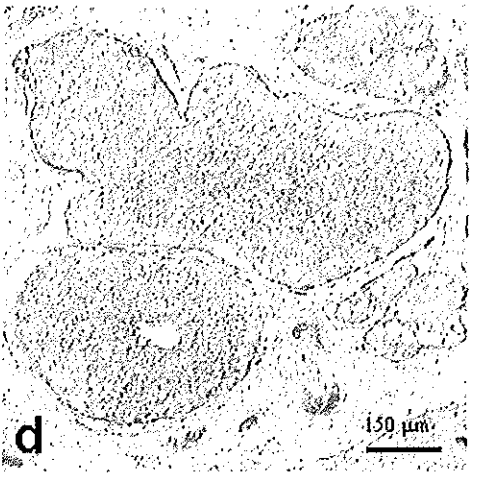
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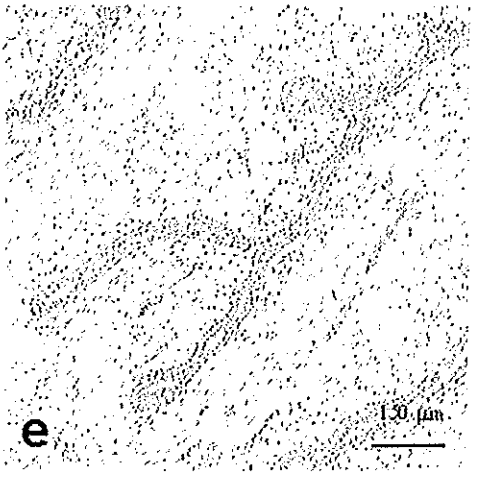
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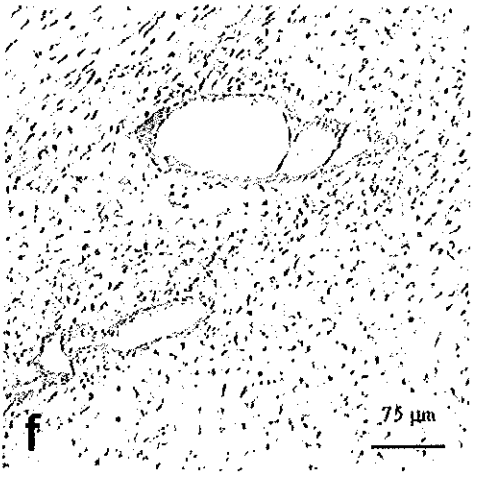
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Table 1. **Immunohistochemical staining (IH) of BCAR1/p130Cas expression in normal tissue and various neoplasms of the breast**

Case	Age Years	Tissue/Tumor type[a]	IH BCAR1/p130Cas				
			Epithelial cells		stromal cells		blood vessels
			Value[b]	int[c]	value[b]	int[c]	int[d]
1	38	Non-malignant	1	+/++	1	0	++
2	51	Non-malignant	1	++	1	0	+++
3	29	Non-malignant	1	++	1	0	++
4	27	Non-malignant	1	+	1	0	++
5	19	Non-malignant	1	++	1	0	++
6	21	Fibroadenoma	1	+	1	0	++
7	53	Myofibroblastoma	0	0	1	0	+++
8	64	Phyllodes	1	+	1	0	++
9	48	Phyllodes	1	++	1	0	+++
10	48	Phyllodes	1	+	1	0	+
11	21	Phyllodes	1	++	1	0	++
12	45	Phyllodes	1	++	1	0	++

[a] Histological classification of the tissue/tumor type; [b] Presence of epithelial or stromal cells, 0=not present, 1=present; [c] Intensity of the estimated average staining intensity of BCAR1/p130Cas positive epithelial cells or stromal cells, 0=none, +=weak, ++=intermediate and +++=strong; [d] Estimated average staining intensity of BCAR1/p130Cas in the blood vessels, 0=none, +=weak, ++=intermediate and +++=strong.

Results

BCAR1/p130Cas in benign breast tissue

Immunohistochemical staining of five non-malignant breast tissues using the αp130Cas TL antibody showed clear immunoreactivity of the epithelial structures for BCAR1/p130Cas (Figure 1a and Table 1). In the normal ducts and lobules, the BCAR1/p130Cas protein is localized in the cytoplasm but not in the nuclei of the luminal and myoepithelial cells (Figure 1b). In some cases the immunostain was stronger in the myoepithelial cells lining the ducts (Figure 1b), although in other cases equal or stronger staining of the luminal cell was observed. In addition to

the epithelial cells, BCAR1/p130Cas was also detected in the blood vessels throughout the interductal stroma. Notably the endothelial cells and smooth muscle cells of the blood vessels stained strongly. In contrast no immunoreactivity for BCAR1/p130Cas was seen in the stromal cells of the connective tissue. The intensity of the BCAR1/p130Cas staining of the epithelial and blood vessel cells was evaluated and is represented in Table 1. No staining was observed in the negative controls in which preimmune mouse IgG1 was used instead of αp130Cas antibody.

BCAR1/p130Cas in breast carcinomas

The localization and distribution pattern of the BCAR1/p130Cas protein in 18 breast carcinomas was evaluated immunohistochemically using the αp130Cas TL antibody (Table 2). The epithelial component in the carcinoma sections was immunoreactive to BCAR1/p130Cas and the staining of these carcinoma cells generally was homogeneous and diffusely cytoplasmic (Figure 1c and 1d). In most carcinoma sections the majority of tumor cells was immunoreactive to BCAR1/p130Cas, but in some cases also areas of BCAR1/p130Cas negative carcinoma cells were present. However, all carcinomas, except one, showed BCAR1/p130Cas expression, but considerable variation was observed in the intensity of the staining (Table 2). BCAR1/p130Cas-positive blood vessels were uniformly found in the carcinomas (Table 2), showing intense staining of the smooth muscle and endothelial cells. Detailed examination of the sections containing non-neoplastic tissue adjacent to carcinoma revealed clear cytoplasmatic staining in the luminal and myoepithelial cells of the non-malignant epithelial structures. In these sections the staining of the non-malignant epithelial cells and the tumor cells was of comparable intensity, but in a few cases the non-malignant epithelial cells exhibited stronger staining than the tumor cells. No or occasionally very weak immunoreactivity for BCAR1/p130Cas were detected in the connective tissue stroma cells adjacent to the epithelial (tumor) cells. The antibody of different isotype (8G4-E8), also raised against the carboxy terminal part of rat p130Cas, was specific for BCAR1/p130Cas protein in our

Table 2. **Immunohistochemical staining (IH) of BCAR1/p130Cas protein in primary breast carcinomas**

Case	Age years	Tumor type[a]	Tumor area[b]	IH BCAR1/p130Cas					WB[g] int
				carcinoma cells		non-malignant epithelial cells		blood vessels	
				% [c]	int[d]	value[e]	int[d]	int[f]	
1	52	Invasive ductal carcinoma	3	2	+	0	0	+++	2
2	67	Invasive ductal carcinoma	2	4	+	0	0	+++	1
3	63	Invasive ductal carcinoma	3	5	+++	0	0	+++	3
4	50	Invasive ductal carcinoma	3	2	+	0	0	+++	2
5	74	Invasive ductal carcinoma	2	5	++	0	0	+++	2
6	50	Invasive ductal carcinoma	2	4	++	0	0	+++	2
7	53	Invasive ductal carcinoma NOS	3	4	+	1	+	+++	2
8	71	Invasive ductal carcinoma NOS	4	5	+++	0	0	+++	2
9	48	Ductal carcinoma NOS	3	5	++	1	++	+++	2
10	49	Intraductal carcinoma comedo	3	5	+++	1	+++	+++	3
11	59	Invasive ductal carcinoma, DCIS	3	5	+	1	++	+++	1
12	70	Invasive ductal carcinoma, Lobular carcinoma	2	4	++	1	++	+++	2
13	48	Invasive ductal carcinoma, Lobular carcinoma	3	4	+	1	+++	+++	2
14	52	Lobular CIS, DCIS, Colloid carcinoma	3	5	++	0	0	+++	1
15	77	Invasive lobular carcinoma	1	0	0	1	++	+++	0
16	78	Invasive lobular carcinoma	3	4	+	1	++	+++	2
17	80	Invasive lobular carcinoma	4	5	+	1	+	+++	3
18	64	Papillary carcinoma	4	5	+	0	0	+++	3

[a] Histological classification of the carcinoma, NOS=not otherwise specified, CIS=carcinoma *in situ*, DCIS=ductal carcinoma *in situ*; [b] Percentage of the section occupied by the tumor, 1=<25%, 2=25-50%, 3=50-75%, 4=75-100%; [c] Percentage BCAR1/p130Cas positive carcinoma cells, 0=0%, 1=1-5%, 2=6-25%, 3=26-50%, 4=51-75%, 5=76-100%; [d] Intensity of the estimated average staining intensity of BCAR1/p130Cas positive epithelial (tumor)cells, 0=none, +=weak, ++=intermediate and +++=strong; [e] Presence of non-malignant epithelial cells; 0=not present; 1=present; [f] Estimated staining intensity of BCAR1/p130Cas in the blood vessels, 0=none, +=weak, ++=intermediate and +++=strong; [g] Western blot analysis of BCAR1/p130Cas expression, 0= no expression, 1= low expression, 2= intermediate expression, 3= high expression

Western blot analysis and showed a corresponding but weaker staining pattern for each of the three carcinomas analyzed compared to the αp130Cas TL antibody (results not shown). Additionally to the immunohistochemistry on tissue sections we performed Western blot analysis on tissue extracts of these carcinoma tissues and determined semi-quantitatively the BCAR1/p130Cas levels (Table 2). The BCAR1/p130Cas protein was expressed at varying levels in the carcinomas as shown in Figure 2 and depicted in Table 2. The Western blot intensity scoreswere generally similar to the BCAR1/p130Cas immunohistochemistry results (staining intensity, tumor area and proportion of positive cells).

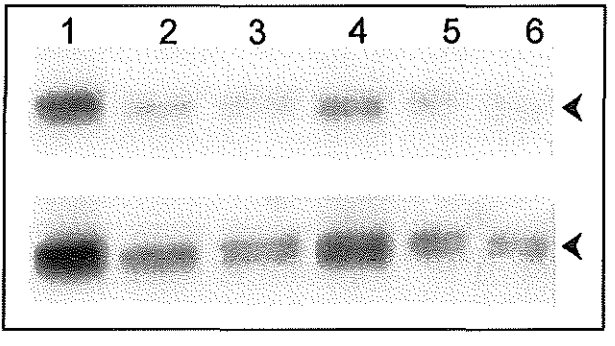

Figure 2. **Western blot analysis of tissue lysate of human breast carcinomas.** Three micrograms of tissue lysate from six primary breast carcinomas (lane 1: carcinoma 3; lane 2: carcinoma 8; lane 3: carcinoma 13; lane 4: carcinoma 17; lane 5: carcinoma 12; lane 6: carcinoma 9) were examined by Western blot analysis with αp130Cas TL. Two exposure times of the relevant part of the blot are shown with the position of the BCAR1/p130Cas band (approximately 116 kD) indicated by the arrowhead. Short (top) and long (bottom) exposures were used to classify BCAR1/p130Cas expression as 1 (lane 6), 2 (lane 2, 3, and 5) or 3 (lane 1 and 4).

BCAR1/p130Cas in breast tumors of mesodermal origin

Five phyllodes tumors, a fibroadenoma and a myofibroblastoma were analyzed immunohistochemically using the αp130Cas TL antibody (Table 1). The areas examined were predominantly of mesenchymal origin but in all cases, but one, ductal structures were also present. The hypercellular stroma did not stain for BCAR1/p130Cas in any of the sections analyzed. In the epithelial structures, immunoreactivity for BCAR1/p130Cas was demonstrated in the luminal and

myoepithelial cells (Figure 1e and 1f). The blood vessels vessels present in these tumor tissues of stromal origin were all BCAR1/p130Cas positive (Table 1).

Discussion

Previously we have shown that an increasing dosage of the BCAR1/p130Cas protein, determined in the cytosolic fraction of primary breast carcinomas, was associated with an early recurrence of the disease (*16*). Our current study demonstrates that the BCAR1/p130Cas protein is localized in the epithelial cells and the blood vessels of breast tissue. The specificity of this BCAR1/p130Cas staining pattern was confirmed by another monoclonal antibody directed against the BCAR1/p130Cas protein. The present findings demonstrate that high BCAR1/p130Cas expression levels on Western blots correspond with relatively large areas of intense staining of malignant epithelial cells and staining of the vascular compartment. This suggests that an increased concentration of BCAR1/p130Cas in the tumor may reflect a more aggressive tumor subtype. Such a mechanism is supported by the reported role of BCAR1/p130Cas in cell migration and invasion (*6-9*), properties which are implicated in disease recurrence. However, it should be noted that many functions of BCAR1/p130Cas have also been found to be dependent on protein tyrosine phosphorylation of the protein (*10,20*). This type of modulation of BCAR1/p130Cas function in relation to breast cancer recurrence and anti-estrogen resistance remains to be investigated *in vitro* and in patients samples.

The BCAR1/p130Cas protein is present in the cytoplasm of the epithelial cells and not in the nucleus. The connective tissue stroma, including the fibroblasts, of the breast tissues analyzed was not or hardly immunoreactive to BCAR1/p130Cas. Neither did the hypercellular stroma of the immunohistochemically analyzed phyllodes tumors and the fibroadenoma stain for BCAR1/p130Cas. However, in several *in vitro* studies this protein has been described to be abundantly expressed in cultured fibroblasts of different species including human fibroblasts (*21*). We did not observe a correlation between BCAR1/p130Cas expression and the histologically type of carcinoma in this

series of breast tumor samples. Both ductal and lobular carcinomas exhibited BCAR1/p130Cas expression in the carcinoma cells.

In our Western blot studies, BCAR1/p130Cas protein was detected in cytosols of both estrogen receptor (ER)- negative and ER-positive tumors, with increasing levels in the latter group (*16*). The present study demonstrates that the majority of luminal epithelial cells as well as the myoepithelial cells are BCAR1/p130Cas-positive. These tissues have not been further classified according to the pattern of ER staining, since this was not the subject of the current study. Previous double-staining experiments on breast tissues by Van Agthoven *et al* (*22*) have demonstrated that the majority of the luminal epithelial cells are ER-positive/epidermal growth factor (EGFR)-negative whereas the myoepithelial cells were ER-negative/EGFR-positive. All together, these results suggest that BCAR1/p130Cas is present in both ER-positive as ER-negative cells. Currently, double staining experiments for ER and BCAR1/p130Cas are in progress in our laboratory.

Interestingly, we found staining for BCAR1/p130Cas in the endothelial and smooth muscle cells of the arteries in benign and malignant breast tissue. This observation is in agreement with *in vitro* studies showing that p130Cas is expressed in vascular smooth muscle cells and is involved in functions such as contraction, migration, and proliferation (*23,24*). Moreover, studies on p130Cas-deficient mice have identified a role for BCAR1/p130Cas in the maintenance of blood vessel integrity during embryogenesis (*13*).

The variable level of BCAR1/p130Cas expression observed in the large series of breast carcinoma cytosols (*16*) were confirmed in the present study (Figure 2 and Table 2). In contrast, expression of BCAR1/p130Cas in normal breast tissue lysates was not detected by Western blot analysis (*16*), although immunohistochemical analysis demonstrated the presence of BCAR1/p130Cas-positive epithelial cells and vessels (Figure 1a and 1b). These differing findings may be explained by the following observations: i) the varying proportion of epithelial cells present in the tissue, and ii) the varying BCAR1/p130Cas staining intensities of the epithelial cells. We conclude that, as a result of the low

proportion of BCAR1/p130Cas-expressing epithelial cells which are contributing to the total protein content in the extracts of the non-malignant breast tissues, its level was below the detection threshold of the immunoblot procedure. Immunohistochemical localization with the αp130Cas antibodies is, however, well suited to examine the presence of BCAR1/p130Cas positivity in normal breast tissue.

The occurrence of BCAR1/p130Cas in both non-malignant and malignant breast epithelial cells indicates that this protein is probably not required for tumor formation. However, our previous study demonstrate that high BCAR1/p130Cas expression is a hallmark of rapid breast tumor recurrence and intrinsic resistance to tamoxifen treatment. The underlying molecular mechanism and signaling events involved in these processes in breast epithelial cells remain to be resolved.

Acknowledgement

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CHAPTER 3

General Discussion and Summary

Breast cancer and tamoxifen resistance

The development and progression of breast cancer is a multistep process, and breast cancer is thought to take place via non-invasive and invasive stages, frequently succeeded by metastatic cancer. In the development and progression of breast cancer a prominent role is played by the estrogens that interact with the nuclear estrogen receptorα (ERα) stimulating tumor growth. Endocrine treatment has been designed to antagonize the effects of estrogens in the tumor cells. The anti-estrogen tamoxifen competitively blocks binding of estrogens to ERα and thus prevents the transcriptional activation of genes required for tumor growth. Endocrine therapy with tamoxifen is the first-line endocrine treatment option for the majority of patients with metastatic breast cancer and is an integral component of adjuvant therapy for many patients with early-stage breast cancer without evidence of distant metastases. The extent of disease at diagnosis (e.g. tumor size and the presence or absence of lymph node- and distant-metastases) is the major predictor of survival. In addition, the presence of the ER in the primary tumor has been proven to be important in predicting improved disease-free survival on adjuvant hormonal treatment and in predicting response of recurrences under tamoxifen therapy. About half of the patients receiving first-line tamoxifen therapy show a favorable response. All patients responding to treatment will eventually relapse. Patients with ER-positive advanced breast cancer who are resistant to endocrine therapy can be divided into two groups: i) those showing complete resistance to all endocrine treatments can be considered as intrinsically resistant, and ii) those who respond to endocrine therapy initially, and develop resistance under therapy (acquired resistance) (*1*).

The mechanisms involved in intrinsic and acquired tamoxifen resistance are not well understood. (Epi)genetic alterations in the tumor cells may be responsible for hormone-independent growth and intrinsic resistance, and contribute to the development of acquired tamoxifen resistance in breast cancer. To search for genetic factors involved in tamoxifen resistance, an *in vitro* model was generated by employing retroviral insertional mutagenesis. Retroviral integration in the host genome results in local alterations and may affect local gene expression. At the

same time the provirus serves as a genetic tag that enables the isolation of the genomic integration sites and the characterization of genes involved in tamoxifen resistance. The first common integration site identified in this *in vitro* model was termed the *BCAR1* locus. The work described in this thesis focusses on three questions: (i) which gene in the *BCAR1* locus is responsible for tamoxifen resistance *in vitro* (Chapter 2.1); (ii) what is the involvement of BCAR1 in clinical tamoxifen resistance (Chapter 2.2 and 2.4); (iii) in which cells of breast tissue is the BCAR1 protein localized (Chapter 2.3)?

BCAR1 is the human homologue of the adapter protein p130Cas, and is involved in anti-estrogen resistance in breast cancer cells

Chapter 2.1 describes the isolation and characterization of a candidate *BCAR1* gene in the *BCAR1* locus by exon-trapping and cDNA library screening. Northern analysis demonstrated widespread expression of the *BCAR1* gene that proved to be the human homologue of the rat gene for the p130 Crk-associated protein (p130Cas). Human BCAR1 is therefore termed BCAR1/p130Cas. Transfection experiments demonstrated the functional involvement of this candidate *BCAR1/p130Cas* gene in anti-estrogen resistance in human breast cancer cells *in vitro*. These results showed that this gene is the crucial gene in the *BCAR1* locus that is dominantly responsible for tamoxifen-resistant cell proliferation.

The *BCAR1/p130Cas* gene is localized at human chromosome 16q22-23. Over recent years, frequent losses of chromosomal regions at 16q have been reported, indicating the inactivation of a yet unknown tumor suppressor gene (*2,3*). However, no loss of regions overlapping the *BCAR1/p130Cas* gene has been demonstrated. Moreover, the *BCAR1/p130Cas* gene is not assumed to be a tumor suppressor gene, since enhanced expression of BCAR1/p130Cas causes anti-estrogen resistant cell proliferation.

An important issue for further research is the identification of the specific protein-domain(s) and the protein-protein interactions of BCAR1/p130Cas that are essential for anti-estrogen resistant proliferation of human breast epithelial cells: BCAR1/p130Cas is known to associate with many proteins through its SH3

domain and the various SH2-binding sites. Alignment of the sequences of the BCAR1/p130Cas, HEF1/Cas-L, and Efs/Sin family members has demonstrated a considerable sequence homology in the SH3 domain, and indicated that the carboxy-terminal domain constitutes one of the most highly conserved regions of the family. Domain-swapping between BCAR1/p130Cas and these structural related family members HEF1/Cas-L and Efs/Sin may help to define regions and/or interacting proteins that are important in mediating BCAR1/p130Cas-stimulated cell proliferation.

High level of BCAR1/p130Cas protein in primary breast cancer predicts poor prognosis

In Chapter 2.2 the clinical relevance of BCAR1/p130Cas in breast cancer was evaluated, applying Western blot analysis on cytosols of over 900 primary breast carcinomas. The level of BCAR1/p130cas expression was determined for the carcinomas and was correlated with various characteristics of the patients and their disease for clinically relevant associations. This established an independent prognostic role for BCAR1/p130Cas in patients with primary or metastatic breast cancer. A high level of BCAR1/p130Cas was found in human primary breast carcinomas positive for ER and PgR, and was shown to be related to a significantly higher recurrence rate. This hints at a more aggressive behavior of tumors with high BCAR1/p130Cas levels and thus a higher risk of early metastatic spread of tumor cells. However, no association between lymph node status and BCAR1/p130Cas level was observed, and relatively low levels of BCAR1/p130Cas are found in poorly differentiated tumors and higher levels in older patients.

In several *in vitro* studies the rat p130Cas protein has been shown to be involved in the invasion, motility, and migration machinery of cells (*4-7*), processes required for the metastatic capacity of tumor cells, thus indicating the mechanistic association of p130Cas with metastasis. Additional support is provided by the finding that p130Cas indirectly reverses the inhibition of cell invasion and migration effectuated by the tumor suppressor PTEN (*8*). Recently, p130Cas was

demonstrated to upregulate the transcription of several matrix metalloproteinases (MMPs) via its interaction with CIZ, Cas-interacting zinc finger protein (*9*). Studies performed over the last decade have established a crucial role for MMPs in tumor invasion of tumor cells, since increased expression or activity of MMPs has been linked to malignancy and invasion of tumor cells (*10*). Angiogenesis forms another essential process in tumor growth and metastasis and is brought about by a paracrine interplay between tumor cells and endothelial cells. The vascular endothelial growth factor (VEGF) is produced by the tumor, whereas its receptor (VEGFR) is expressed on proliferating endothelial cells. VEGF stimulation has been reported to cause increased tyrosine phosphorylation of p130Cas and FAK via Src kinase, again indicating a function for p130Cas as a signaling molecule in tumor proliferation and migration (*11*).

In conclusion, metastasis is the most lethal attribute of a cancer and requires the interplay of a number of gene products. Markers capable of identifying patients at risk for metastatic spread, and/or better treatment targeted to eradicate metastatic tumor deposits both could have a dramatic impact on the overall survival of breast cancer patients and might decrease the likelihood of therapy-resistance of tumors. However, whether BCAR1/p130Cas could be a useful marker for disease recurrence requires a better understanding of its involvement in the metastatic process of breast cancer.

A high BCAR1/p130Cas level is associated with intrinsic resistance but not with acquired resistance to tamoxifen treatment

For 268 patients with recurrent disease the BCAR1/p130Cas protein levels of the primary carcinomas were tested against the efficacy of first-line treatment with tamoxifen for recurrent or metastatic breast cancer (Chapter 2.2). High expression of BCAR1/p130Cas in the predominantly ER-positive primary breast carcinomas (11% of patients) was shown to predict a greater risk of intrinsic resistance (no response) to first-line tamoxifen therapy. This established an independent predictive role for BCAR1/p130Cas in patients with primary or metastatic breast cancer.

The problem of acquired tamoxifen resistance, which could result from the selection of cells whose growth is not inhibited by the anti-estrogen, is an important challenge in cancer biology. Chapter 2.4 focuses on the role of BCAR1/p130Cas in acquisition of anti-estrogen resistance. The BCAR1/p130Cas protein level was quantitatively analyzed by chemiluminescent Western blot analysis of cytosols of untreated primary breast carcinomas and of carcinomas that initially responded to tamoxifen treatment and subsequently progressed. No significant difference was detected in the median levels of BCAR1/p130Cas expression between untreated and acquired tamoxifen-resistant carcinomas. In conclusion, tumor progression towards tamoxifen-resistance is unlikely to be the result of a generally occurring upregulation of BCAR1/p130Cas expression in the tumor cells. However, the limited number of available patient specimen precludes a definite conclusion whether increased BCAR1/p130Cas expression might be responsible for acquired tamoxifen resistance in a subgroup of patients. Nevertheless, our results suggest that a high BCAR1/p130Cas protein level is a hallmark of breast carcinomas with intrinsic rather than acquired resistance to tamoxifen. So carcinomas with high BCAR1/p130Cas levels being intrinsically resistant to anti-estrogens probably bypass the pathway of ER-regulated cell proliferation and use an alternative signal cascade involving BCAR1/p130Cas. Indeed our *in vitro* studies (Chapter 2.1) showed that overexpression of the *BCAR1/p130Cas* gene in ER-positive cells conferred resistance to anti-estrogens independent of the ER-pathway.

The BCAR1/p130Cas protein is expressed in the epithelial and vascular component of non-malignant and malignant breast tissue

The data presented in Chapter 2.3 demonstrate by immunohistochemical methods the localization pattern of the BCAR1/p130Cas protein in primary breast carcinomas, in tumors of stromal origin, and in non-neoplastic tissues using antibodies raised against the rat p130Cas protein. The BCAR1/p130Cas protein was detected in the epithelial cells and the vascular component of the non-malignant and malignant breast tissues analyzed. In contrast, the stromal cells in

non-malignant tissues and the hypercellular stroma in the tumor tissues hardly showed staining for BCAR1/p130Cas. Immunohistochemistry demonstrated a variable intensity of BCAR1/p130Cas staining and a variation in the proportion of BCAR1/p130Cas-positive carcinoma cells for the different breast carcinomas. Western blot analysis of the carcinomas resulted in scores that were generally similar to the immunohistochemistry results of the carcinoma tissues. Also for the non-malignant breast tissues a variable BCAR1/p130Cas expression was detected in the epithelial cells. However, the relatively low number of epithelial cells present in these tissues precludes BCAR1/p130Cas detection on Western blots. The variation of expression in the malignant cells may reflect a specific feature of breast carcinoma tissue.

An urgent question arising as a consequence of our results described in Chapter 2.2 is whether BCAR1/p130Cas is co-expressed with ER in breast cancer cells in these breast tissues (*12*). Recently, immunohistochemical colocalization studies for ER and BCAR1/p130Cas have been performed on benign and malignant breast tissue in our laboratory. These experiments demonstrated that co-expression of BCAR1/p130Cas and ER occurs in individual epithelial cells. In addition, BCAR1/p130Cas expression was also observed in ER-negative breast carcinoma cells and in normal myoepithelial cells.

Concluding remarks

The experiments described in this thesis underscore the effectiveness of the *in vitro* model as a strategy to identify genes (and their products) involved in tamoxifen resistance of clinical breast cancer. Moreover, the results obtained indicate that *in vitro* identified proteins may provide useful prognostic information that may be utilized to select patients for individual treatment. The results suggest that BCAR1/p130Cas may play a role in the metastatic potential and growth control of metastatic breast carcinoma in a subgroup of patients. Our results do not support the hypothesis that the BCAR1/p130Cas level *in vivo* is regulated by anti-estrogen therapy. However, a change in biological activity of BCAR1/p130Cas (i.e. phosphorylation status) or one of the downstream targets of

the intracellular BCAR1/p130Cas signaling pathway might be instrumental in stimulating cell proliferation during tamoxifen treatment.

Further insight in the pathways of tamoxifen resistance may be obtained by analysis of gene expression (expression profiling). The recent development of the cDNA array technology offers the opportunity to unravel the complex pattern of gene expression in breast cancer, and possibly in tamoxifen resistance (*13,14*). This technology may be applied on our panel of resistant cell lines to identify the regulatory pathways controlling anti-estrogen resistant cell proliferation.

Identification of critical changes in the signaling pathway involved in tumor progression and tamoxifen resistance will not only provide insight into the genetic and molecular basis of this process, but should also identify targets for new therapies. Clarification of the role of BCAR1/p130Cas might provide an opportunity to utilize the BCAR1/p130Cas signaling pathway as a potential drug target. Drawback for such a treatment would be the ubiquitous expression of BCAR1/p130Cas mRNA in human tissues.

In conclusion, elucidation of the mechanisms and pathways involved in tamoxifen resistance is essential to achieve improved treatment strategies for breast cancer.

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Samenvatting

De ontwikkeling van borstkanker is een meerstaps-proces dat verloopt via een pre-invasief en een invasief stadium, vaak gevolgd door metastasering. Gedurende de progressie van borstkanker neemt het aantal (epi)genetische veranderingen op specifieke genomische loci toe met het stadium van de laesie. Bij de ontwikkeling van borstkanker spelen de oestrogenen een prominente, groeistimulerende rol. Endocriene behandeling is ontwikkeld om het effect van oestrogeen op de tumorcellen te antagoneren. Het anti-oestrogeen tamoxifen blokkeert op competitieve wijze de binding van oestrogeen aan de oestrogeen receptor (ER) en voorkomt zo de transcriptionele activatie van diverse genen die vereist zijn voor tumorgroei. Therapie met tamoxifen is de eerste-lijns endocriene behandelingsoptie voor de meerderheid van de patiënten met uitgezaaide borstkanker. Het wordt ook toegepast als adjuvante therapie bij patiënten met borstkanker zonder bewezen metastasen op afstand. Het stadium van de ziekte op het moment van diagnose (bv. tumorgrootte en de aanwezigheid of afwezigheid van metastasen) is de belangrijkste voorspeller van overleving. Bovendien is gebleken dat aanwezigheid van ER in de primaire tumor een gunstige factor is voor de ziekte-vrije overleving in het geval van adjuvante hormonale behandeling en voor de respons van metastasen op tamoxifen therapie. Minder dan de helft van de patiënten met metastasen die eerste-lijns tamoxifen behandeling ontvangen, toont een objectieve respons. Bij de patiënten die reageren op de behandeling komt de ziekte vroeger of later terug. Dus bij meer dan de helft van de patiënten zijn de ER-positieve tumoren intrinsiek resistent en bij de andere helft van de patienten worden de tumoren resistent onder de behandeling met tamoxifen.

Tot nu toe zijn de mechanismen betrokken bij intrinsieke en verworven resistentie niet goed begrepen. Verondersteld wordt dat (epi)genetische veranderingen in de tumorcellen verantwoordelijk zijn voor hormoon-onafhankelijke groei en intrinsieke resistentie en bijdragen aan de ontwikkeling van verworven tamoxifen-resistentie van borstkanker. Om te onderzoeken welke genetische factoren betrokken zijn bij tamoxifen-resistentie is een *in vitro* model opgezet waarin

gebruik wordt gemaakt van retrovirale insertie mutagenese. Integratie van retrovirussen in het gastheer genoom resulteert in lokale veranderingen die de expressie van genen beïnvloeden. Het geïntegreerde virus dient ook als genetische vlag die de isolatie van de integratieplaats en de karakterisering van het gen dat betrokken is bij tamoxifen resistentie mogelijk maakt. Een integratieplaats, het *BCAR1* locus genoemd, werd reeds eerder in dit *in vitro* systeem geïdentificeerd.
Het werk beschreven in dit proefschrift heeft zich gericht op de volgende drie vragen: (i) welk gen in het *BCAR1* locus is verantwoordelijk voor tamoxifen resistentie *in vitro* (hoofstuk 2.1), (ii) wat is de betrokkenheid van BCAR1 bij tamoxifen resistentie bij patienten (hoofdstuk 2.2 en 2.3) en (iii) waar is het BCAR1 eiwit gelocaliseerd in normaal- en tumorweefsel van de borst (hoofdstuk 2.4)?
Hoofdstuk 2.1 beschrijft de isolatie en karakterisering van een kandidaat *BCAR1* gen in het *BCAR1* locus m.b.v. exon-trapping en screening van een cDNA bank. Northern blot analyse toonde een wijdverspreide expressie van het *BCAR1* gen aan. Het humane *BCAR1* gen bleek homoloog te zijn met het ratten-gen voor het p130-Crk-geassocieerd eiwit (p130Cas). Humaan BCAR1 wordt vanwege deze verwantschap BCAR1/p130Cas genoemd. Transfectie experimenten toonden dat dit kandidaat *BCAR1/p130Cas* gen betrokken is bij anti-oestrogeen resistentie in humane borstkankercellen *in vitro*. Deze studie laat zien dat dit het cruciale gen is in het *BCAR1* locus en dat het dominant verantwoordelijk is voor tamoxifen-resistente celgroei.
In hoofdstuk 2.2 is de klinische relevantie van BCAR1/p130Cas in borstkanker geëvalueerd door toepassing van Western blot analyse op cytosolen van ruim 900 primaire borstcarcinomen. Het niveau van BCAR1/p130Cas expressie in de carcinomen werd gecorreleerd met verschillende karakteristieken van de patiënten en hun ziekte. Hoge expressie van BCAR1/p130Cas in humane borstcarcinomen bleek gepaard te gaan met een significant kortere ziekte-vrije overleving en dit voorspelde een groter risico op intrinsieke resistentie tegen eerste-lijns tamoxifen therapie. Deze studie toonde aan dat BCAR1/p130Cas een onafhankelijke

prognostische betekenis heeft in patiënten met een primaire of gemetastaseerde vorm van borstkanker.

Hoofdstuk 2.3 richt zich op de betrokkenheid van de BCAR1/p130Cas eiwit expressie in de verwerving van anti-oestrogeen resistentie. BCAR1/p130Cas eiwit-niveaus werden kwantitatief bepaald d.m.v. chemiluminescente Western blot analyse van cytosolen van primaire borstcarcinomen en van tamoxifen behandelde carcinomen die initiëel respondeerden op tamoxifen en vervolgens progressie vertoonden. Er werd geen significant verschil aangetoond in mediane niveaus van BCAR1/p130Cas expressie tussen onbehandelde carcinomen en carcinomen met verworven tamoxifen-resistentie. Concluderend, verworven tamoxifen-resistentie is waarschijnlijk in een kleine minderheid van de carcinomen het gevolg van op-regulatie van BCAR1/p130Cas expressie in de carcinoomcellen. Echter, het beperkte aantal beschikbare patiënten voor deze studie sluit een definitieve conclusie betreffende de betrokkenheid van verhoogde BCAR1/p130Cas expressie bij verworven tamoxifen-resistentie van de tumorcellen uit. De resultaten suggereren dat hoge BCAR1/p130Cas eiwit-niveaus van de borstcarcinomen een kenmerk zijn voor intrinsieke resistentie maar in de verwerving van tamoxifen-resistentie een beperkte rol spelen.

In de studie beschreven in hoofdstuk 2.4 werden m.b.v. immunohistochemische methoden de localisatie en het expressie-niveau van het BCAR1/p130Cas eiwit aangetoond in primaire borstcarcinomen, in tumoren van stromale oorsprong, en in niet-neoplastisch borstweefsel. Hiervoor werd gebruik gemaakt van antilichamen opgewekt tegen ratten-p130Cas eiwit. Het BCAR1/p130Cas eiwit komt tot expressie in epitheliale cellen en de vaten van zowel normaal weefsel als van tumoren van de borst. In tegenstelling hiermee vertoonden de bindweefselcellen in het normale en het tumorweefsel geen aankleuring voor BCAR1/p130Cas. De intensiteit van de aankleuring voor BCAR1/p130Cas was variabel, bovendien verschilde het percentage BCAR1/p130Cas positieve tumorcellen in de verschillende borstcarcinomen. Ook in het normale borstweefsel werd een variabele BCAR1/p130Cas expressie gedetecteerd in de epitheliale

cellen. De variatie in de expressie in de maligne cellen zou verschillen in het klinisch gedrag van tumoren kunnen verklaren.

De studie beschreven in dit proefschrift demonstreert de effectiviteit van het *in vitro* systeem voor de identificatie van genen (en hun producten) die betrokken zijn bij tamoxifen resistentie bij patiënten met borstkanker. Bovendien geven de verkregen resultaten aan dat *in vitro* geïdentificeerde eiwitten waardevolle prognostische informatie kunnen geven die gebruikt zou kunnen worden bij de behandeling van individuele patiënten. Opheldering van de moleculaire mechanismen van tamoxifen-resistentie is een essentiële stap in de verdere ontwikkeling van behandelingsstrategieën die specifiek gericht zijn op de genetische veranderingen in borstkanker.

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Curriculum Vitae

Silvia van der Flier is op 23 augustus 1966 geboren te Rotterdam. In 1984 behaalde zij het HAVO diploma aan de christelijke scholengemeenschap Comenius te Capelle a/d IJssel. In hetzelfde jaar begon zij met het hoger laboratorium onderwijs aan het Van Leeuwenhoek Instituut in Delft. Tijdens een onderzoeksstage bij de vakgroep Cytochemie & Cytometrie van de Rijksuniversiteit Leiden (dr. P.M. Nederlof) werd onderzoek verricht aan de ontwikkeling van methoden voor kwantitatieve en multipele fluorescentie *in situ* hybridisatie methoden. Het diploma in de studierichting biotechnologie werd behaald in 1988 waarna zij gedurende 2 jaar als analiste in de onderzoeksgroep van prof. dr. H.J. Tanke en prof. dr. A.K. Raap bij de vakgroep Cytochemie & Cytochemie bleef werken.

In 1990 begon zij met de studie scheikunde aan de Rijksuniversiteit Leiden. Gedurende deze studie werkte zij 1½ dag per week als analiste in de onderzoeksgroep van dr. P.I. Schrier bij de vakgroep Klinische Oncologie van het Academisch Ziekenhuis Leiden. Tijdens de onderzoeksstage bij de afdeling Moleculaire Plantkunde van de Rijksuniversiteit Leiden (dr. C.L. Diaz) werd een studie verricht aan de lectine-samenstelling en -verdeling tijdens de ontwikkeling van *Pisum sativum*. In 1993 werd het doctoraalexamen scheikunde behaald, waarna zij een half jaar door Azië reisde. In 1994 was zij als analiste korte tijd werkzaam bij de afdeling Klinische Genetica van de Erasmus Universiteit Rotterdam.

Half oktober 1994 werd aangevangen met het promotieonderzoek bij de afdeling Pathologie/sectie Moleculaire Biologie in het Josephine Nefkens Instituut. Hier voerde zij onder begeleiding van dr. L.C.J Dorssers het in dit proefschrift beschreven onderzoek uit.

Sinds mei 1999 is zij als regulatory affairs scientist werkzaam bij N.V. Organon te Oss.

Nawoord

Terugkijkend op de afgelopen jaren 'promotie-onderzoek' kan ik niet anders dan tot de conclusie komen dat deze periode in mijn leven leerzaam, karaktervormend, en in velerlei opzichten bijzonder is geweest. Dat had veelal te maken met de collegialiteit en betrokkenheid van veel personen. Daarom op deze, veelal meest gelezen, laatste pagina van het proefschrift een speciaal woord van dank.

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