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Estrogen receptor co-regulators as prognostic and predictive markers of endocrine therapy in early breast cancer: the role of SMRT and p160 family

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FOREWORD

This work was carried out during my appointment as postdoctoral associate at the Lester and Sue Smith Breast Center at Baylor College of Medicine, Houston (TX), under the supervision of Drs Carolina Gutierrez and C Kent Osborne and it derives from two different projects. The first, which analyzes the role of p160 family as prognostic and predictive markers of endocrine therapy response in breast cancer, is a collaboration between the groups of Dr. Steffi Oesterreich from Baylor College of Medicine and Prof. John MS Bartlett from the Edinburgh Cancer Research Centre. The second, which analyzes the role of the ER co-repressor SMRT as prognostic and predictive marker of tamoxifen response, is a collaboration between the groups of Dr. Steffi Oesterreich and Dr. Carolyn Smith from Baylor College of Medicine.

ABSTRACT

Background: The estrogen receptor (ER) signaling pathway is the dominant driver of cell proliferation and survival in the majority of human breast cancers. Not surprisingly, endocrine treatment, such as the anti-estrogen tamoxifen, represents the most effective and widely used therapy for ER positive breast cancer patients. Unfortunately not all patients respond to endocrine treatment and a wide proportion of patients ultimately develop resistance and die. Selecting patients with an increased risk of recurrence and identifying those that might benefit from a particular therapy is of great value in order to personalize breast cancer therapies. A minority of breast cancers does not express ER and displays features of aggressiveness and poor prognosis. Prognostic markers are urgently needed for this subset of patients as well. The p160 family of ER co-activator is composed of three different members: SRC1, SRC2 and AIB1. SRC1 and AIB1 are frequently overexpressed in breast cancer and appear to be linked to hormone resistance, particularly in HER2 positive breast cancer. SMRT is an ER co-repressor that has been implicated in tamoxifen resistance. Data on p160 family members and SMRT expression in human breast cancer samples and its prognostic and predictive significance in endocrine treated patients are controversial or lacking altogether. Moreover, the role of these co-regulators in ER negative disease is poorly understood.

Methods: SRC1, SRC2, AIB1 and SMRT expression was determined by immunohistochemistry on tissue microarrays derived from two fully documented cohorts of 1812 and 1424 patients.

Results: HER2 and AIB1 dual-positive tumors were associated with markedly worse outcome compared to tumors overexpressing either HER2 or AIB1 alone, irrespective of ER status. In ER negative disease both SRC1 and AIB1 were linked to early relapse and death. Additionally, we found that co-expression of two or more SRCs were significantly associated with worse outcome in ER positive endocrine-treated patients. However, expression of any SRC alone was not a significant predictor of resistance to endocrine therapy. Low nuclear SMRT expression was associated with a significantly better outcome in untreated patients but not in tamoxifen-treated patients.

Conclusions: The SRC family of ER co-activators and nuclear SMRT are markers of early relapse in both ER negative and ER positive breast cancer. Evaluation of multiple markers co-expression (i.e. AIB1/HER2, multiple SRCs) rather than single markers allows a better assessment of breast cancer prognosis.

INTRODUCTION

Breast cancer is the most common cancer of women, affecting one in eight women in the western world. Breast cancer alone accounts for 28% (207,090) of all new cancer cases among women in the United States (1) with an estimated 1.4 million new breast cancer cases worldwide each year (2). Mortality from breast cancer has been dramatically reduced mainly as a consequence of the widespread and early application of adjuvant systemic therapy and of early detection due to screening mammography. However breast cancer remains the leading cause of cancer deaths, accounting for approximately 502,000 deaths per year worldwide (3;4). Bio-molecular features of breast cancer such as estrogen receptor (ER), progesterone receptor (PR), epidermal growth factor receptor 2 (HER2) and proliferation index ki-67 are routinely assessed in clinical practice in order to identify patients that are most likely to recur or that might benefit from endocrine and HER2-target therapies. However such prognostic and predictive factors are less than ideal, resulting in inefficient administration of therapy that is sometimes not needed or needed but ineffective. Therefore there is an urgent need to identify new biomarkers in order to personalize breast cancer therapies.

In this thesis I will focus on the role of ER co-regulators as prognostic and predictive markers of endocrine therapy in early breast cancer.

Breast cancer was first hypothesized to be an hormone-dependent disease in 1896, when the British physician George Beatson demonstrated that oophorectomy induced regression of mammary tumors in a subset of premenopausal patients (5). Since then, a variety of clinical and epidemiological observations, with support from cell-culture studies have further proved the involvement of estrogens in the development and/or progression of the disease.

ER structure and signaling

Estrogen mediates its biological effects in target tissues by binding to specific intracellular receptor proteins called estrogen receptor α (ERα) and estrogen receptor β (ERβ) (6;7). ERα was first isolated in the late 1960s and the corresponding gene cloned in the late 1980s (8). A decade later, in 1996, ERβ was cloned (9). Both ERα and ERβ are members of the nuclear hormone receptor superfamily. This includes steroid hormone receptors for progestins, glucocorticoids, mineralcorticoids, androgens, and non-hormone receptors for thyroid hormones, retinoids, and Vitamin D as well as numerous orphan receptors for which no ligand has been identified (10). ERα and ERβ share a high level of sequence homology, conservation of three-dimensional structure and protein domains. ERα, from now on referred as ER, is the most common in the breast and

also the most widely studied. Its structure is composed of six distinct domains, named A to F (11):

1. The A/B domain, located in the amino-terminal portion of the receptor, encodes the activation function 1 (AF1) domain, a region of the receptor involved in protein-protein interactions and transcriptional activation of target gene expression, largely in a hormone independent manner.

2. Domain C encodes the DNA binding domain (DBD), a highly conserved region of the receptor that consists of two functionally distinct zinc-finger motifs. The DBD is responsible for the specific binding of the receptor to the estrogen response element (ERE). It is also responsible for the dimerization of the receptor, allowing the formation of homo- and hetero-dimers.

3. Domain D, also known as hinge region, separates the DBD and the ligand-binding domain. This highly flexible region is important for receptor dimerization. It also contains several sites known to undergo post-translational modifications (such as phosphorylation).

4. Domain E/F, located in the carboxy-terminal portion of the receptors, encodes the ligand-binding domain (LBD). This region contains 12 α-helices, which form a hydrophobic pocket responsible for the ligand binding. Within the LBD resides a second transcriptional activation function domain (AF-2), which is a hormone dependent domain that activates transcription in response to estrogen. AF2 also represents an interaction site for co-activators (12) and co-repressors (13;14), which will be discussed in detail later.

In the classical model of estrogen action, estrogens (E2) diffuse through the membrane, bind to ER and induce a conformational change in the receptor, which leads to the dissociation of heat shock proteins and the formation of receptor homo- or hetero-dimers (15). The E2-ER complex binds to ERE-containing promoter region of estrogen-responsive genes. Upon binding to DNA, E2–ER complexes activate or repress target gene transcription, regulating the proliferation and differentiation of different tissues, including breast, and also promoting breast cancer growth (16).

ER co-regulators

ER transcriptional activity is modulated by a class of proteins, named co-regulators, which includes co-activators and co-repressors (17). Co-activators are

molecules that are recruited by DNA-binding transcription factors to enhance transcription, while co-repressors are molecules that are recruited by transcription factors to repress transcription (18). Co-regulators exist and function in large multiprotein complexes (19).

Co-activators are recruited to target genes in an ordered sequence to enhance transcription by providing the many enzymatic capacities required for control of enhancer-dependent gene expression (20). Co-activator complexes promote transcription by four main mechanisms:

1. Recruiting acetyl-transferase to relax chromatin structure;
2. Recruiting DNA remodeling complexes to unwind the chromatin;
3. Promoting initiation of transcription, elongation of RNA chains and mRNA splicing;
4. Promoting proteolytic termination of the transcriptional response (21;22).

Surprisingly, recent reports show that co-activators can also influence cellular reactions outside the nucleus such as mRNA translation, mitochondrial function, and motility (23).

Co-repressors, on the other hand, seems to function in a completely reverse manner. In particular, they inhibit transcription by several distinct mechanisms, including:

1. Recruiting histone deacetylase complexes to condense DNA;
2. Forming inhibitory complexes with the pre-initiation complex (24;25);
3. Competing with activator proteins for DNA binding and sequestering such activators;
4. DNA methylation (26).

ER Co-activators: The p160/SRC family

The p160 (steroid receptor co-activator- SRC) family of co-activators is one of the most extensively studied families of nuclear receptor co-activators (27;28), consisting of three members:

1. SRC1 (also known as RIP160/NCOA1),
2. SRC2 (also known as TIF2/GRIP1/NCOA2)
3. SRC3 (also known as AIB1/pCIP/RAC3/TRAM1/ACTR/NCOA3, henceforth referred to as AIB1).

All three members share a common structure, which contains three domains (28-30):

1. The amino- terminal basic helix–loop–helix–Per/ARNT/Sim (bHLH–PAS) domain, located at the N-terminal, is the most highly conserved region and mediates protein-protein interactions(31-33).

2. The receptor- interacting domain (RID), centrally located, contains three conserved LXXLL (where L is leucine and X is any amino acid) motifs (34). Numerous evidences suggest that these motifs mediate the interactions with ligand-bounded nuclear receptors (34-36).

3. Two intrinsic transcriptional activation domains (termed AD1 and AD2), located at the C-terminal. AD1 region contains three additional LXXLL motifs. Mutation of one or more of these motifs impairs the interaction of SRCs with the general transcriptional co-integrators CREB-binding protein (CBP) and p300, as well as the activation function of SRCs, indicating that these motifs play a major role in recruiting acetyl-transferases for chromatin remodeling. AD1 does not seem to interact with nuclear receptors (37-39). AD2, responsible for interaction with histone methyltransferases, (40;41), may also be critical for local chromatin remodeling and assembly of the transcriptional machinery around the promoter.

In addition, the C-terminal domains of SRC1 and AIB1 contain histone acetyl-transferase activities.

SRC family members serve as co-activators not only for nuclear receptors, but also for a variety of other transcription factors (30). Accumulated data support a major role of SRCs in the chromatin remodeling and the assembly of general transcription factors through direct and indirect recruitments of other co-activators. The molecular targets of SRCs are numerous. Indeed, SRCs interact with kinases, phosphatases, ubiquitin ligases, small ubiquitin-related modifier (SUMO) ligases, histone acetyl-transferases and histone methyltransferases to modulate gene expression. Thus, SRCs are implicated in a variety of physiological functions and have been suggested to be “master-regulator genes” in the human genome.

Changes in SRCs cellular levels are one way by which cell regulates gene expression. However, several studies have demonstrated that SRCs undergo multiple post-translational modifications such as phosphorylation, ubiquitylation, sumoylation, acetylation and methylation (30;42;43). These modifications, induced by signaling pathways activated by hormones, growth factors and cytokines, play a major role in regulating the transcriptional activity of SRCs.

Deregulated post-translational modifications of SRC molecules have also significant implications in cancer (30;43).

SRC1

SRC1 was first cloned in 1995 (39). Its gene is located in chromosome 2 (p23) (44). SRC1 interacts and enhances a broad range of nuclear receptors, including ER, progesterone receptor (PR), androgen receptor (AR), thyroid hormone receptor (TR), retinoid X receptor (RXR), glucocorticoid receptor (GR), and peroxisome proliferator-activated receptor (PPAR) in a ligand-dependent manner (39;45-47). Additionally, SRC1 can enhance transcriptional activation mediated by other transcription factor such as nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF-kB), SMAD family member 3 (SMAD3), and the transcription factor AP-1 (29;48-50). Despite the fact that SRC1 is widely expressed in many tissues and cell types, studies in knockout mice showed that SRC1 -/- mice exhibit nearly normal growth and fertility (51). However, ovariectomized female SRC1 -/- mice show (i) reduced estrogen-induced uterine growth, (ii) reduced estrogen- and progesterone- dependent uterine decidual response and (iii) reduced mammary gland ductal side branching and alveolar formation, suggesting that SRC1 has a pivotal role in mediating steroid receptor activity (28;52).

SRC1 role in breast cancer has been extensively studied. In vitro studies have demonstrated that SRC1 has a central role in mediating ER-dependent proliferation. Indeed, estrogen induce SRC1 recruitment to the ERE element in breast cancer cell lines and in primary cell cultures derived from patient tumors (53). In MCF-7 breast cancer cells, SRC1 overexpression potentiates estrogen stimulated cell growth (54), whereas a reduction of SRC1 levels reduces estrogen-dependent DNA synthesis and the expression of the estrogen-responsive pS2 gene (55). Furthermore, MCF7 cells lacking SRC1 do not show increased cell proliferation and invasion induced by estrogen. (56). Models of mammary gland tumorigenesis showed that SRC1 is important in breast cancer tumorigenesis and metastasis. Indeed, in transgenic MMTV-PyMT mice, harboring the potent oncogene PyMT under the control of the mammary specific promoter MMTV, SRC1 levels are increased during tumorigenesis and SRC1 deficiency suppresses lung metastasis (57). In addition, a recent study demonstrated that SRC1 promotes breast cancer invasiveness and metastasis by regulating the expression of TWIST1, a master regulator of metastasis and a marker of epithelial-mesenchimal transition, a feature of tumor malignancy and invasiveness (58).

SRC2

The SRC2 gene is located in chromosome 8 (q21) (59). SRC2 interacts with hormone-bound RAR, ER, and PR and enhance AF-1 activity in addition to that of the AF-2 domain (47). SRC2 is widely expressed in many organs and its expression levels differ between cell types and organs. Like SRC1-/- mice, SRC2 -/- mice exhibit nearly normal somatic growth. However, the fertility is significantly reduced in both male and female SRC2 null mice (60) suggesting that SRC2 plays a critical role in reproductive functions.

There are only few studies investigating the role of SRC2 in breast cancer. In vitro studies suggest that, similarly to SRC1, SRC2 reduces estrogen-induced cell proliferation and promotes invasion (55;56).

AIB1

AIB1 was initially identified in an amplified chromosomal 20q region in breast cancer cells (61) and subsequently characterized as a member of the p160 family (62). AIB1 serves as a transcriptional co-activator not only for ER, but also for PR. It can also interact with other transcription factors such as PEA3, E2F1, and AP-1 (28;63;64). Like SRC1 and SRC2, AIB1 is widely expressed. However, unlike SRC1 -/- and SRC2 -/- mice, AIB1 -/- mice display growth retardation, probably due to lower levels of insulin growth factor-I (IGF-I) (65). Moreover, AIB1-/- mice show altered reproductive system. In particular, female mice show reduced levels of estrogens, delay in pubertal development and reduced ovulation capacity. Remarkably, adult female AIB1-/- mice show reduced mammary gland alveolar development in response to estrogen and progesterone.

AIB1 has a central role in breast cancer as demonstrated by numerous in vitro and in vivo studies. In human breast cancer cells, AIB1 functions as an E2F1 co-activator to promote breast cancer cell proliferation (66), mediates insulin-like growth factor I-induced phenotypic changes (67) and enhances estrogen-dependent induction of cyclin D1 expression (68) and epidermal growth factor receptor signaling (69). Additionally, depletion of AIB1 reduces estrogen-mediated cell proliferation and estrogen-dependent colony formation in soft agar (70). In models of mammary gland tumorigenesis, loss of AIB1 suppresses (MMTV)–v-Ha-ras-induced and ERBB2-induced mammary tumor initiation and progression (71;72), makes mammary epithelial cells resistant to DMBA chemical carcinogen induced mammary tumorigenesis (73) and reduces mammary tumor metastasis in the lung (74). On the other hand, overexpression of AIB1 stimulates mammary epithelial proliferation (75), and induces spontaneous mammary adenocarcinomas (76). These important findings define AIB1 as a proto-oncogene.

ER Co-repressors: SMRT/N-CoR

Two nuclear receptor co-repressors have been identified to date: SMRT (silencing mediator for retinoid and thyroid receptors) also known as TRAC-2 and N-CoR (nuclear receptor co-repressor) (77). Both were initially characterized on the basis of their ability to bind members of the thyroid and retinoid receptor family of nuclear receptors in the absence of ligand, and repress transcription (78;79). SMRT and N-CoR are encoded by two distinct loci but share a common molecular architecture which can be divided in two different portion (80;81):

1. A N-terminal portion having three to four distinct transcriptional repression (or silencing) domains (RDs). The RDs are responsible for recruiting additional components of the co-repressor complex, including histone deacetylases, transducin-like protein 1 (TBL-1), G protein pathway suppressor 2 (GPS2), and (possibly) mammalian switch-independent 3 protein (mSin3) (82-86)

2. A C- terminal portion composed of two (SMRT) or three (N-CoR) nuclear receptor interaction domains (NRIDs) (87-90). Each NRID contains a CORNR box (or L/I-X-X-I/V-I) motif that forms the core of the contact surface between the co-repressor and nuclear receptors (91-93).

Initial studies suggested that SMRT/N-CoR co-repressor binding might be limited to nuclear receptors known to repress transcription in the unliganded state, such as T3Rs and RARs (78;94). However, some nuclear receptors display low or no co-repressor binding in the absence of hormone but increase their ability to bind co-repressors in the presence of hormone antagonists: these include ER, PR, AR and GR (95-99). In these receptors, ligands such as tamoxifen or other SERMs (selective endocrine receptor modulators) induce unique conformations that favor co-repressor binding and are distinct from the conformations assumed in the absence of hormone or in response to hormone agonist (12;100;101).

SMRT and N-CoR function by recruiting other proteins, which help mediate the molecular events necessary for repression. Best understood of these downstream recruits are the histone deacetylases, which inhibit transcription by modification of the chromatin template. N-CoR and SMRT can also interact with mSin3, a key co-repressor for many non-receptor transcription factors, (102) and make direct, inhibitory contacts with important components of the general transcriptional machinery such as TFIIB and with TAF30 (24) thereby disrupting formation of the pre-initiation complex and impeding target gene transcription.

SMRT/N-CoR binding is regulated mainly by changes in nuclear receptors induced by hormone agonists or antagonists. In addition, like co-activators, SMRT/N-CoR activity is mediated by post-translational modifications. For example, phosphorylation of the C terminus of SMRT stabilizes co-repressor binding to T3Rs (103). Conversely, negative regulation of SMRT, by its phosphorylation, occurs in response to growth factor receptors operating through a Ras-MEKK1-MEK1 pathway (104;105). Disruption of SMRT gene in knockout mice (SMRT -/- mice) (106;107) is embryonic lethal mainly due to defects in cardiogenesis (107). Knockout of the N-CoR gene (N-CoR -/- mice) is also embryonic lethal (108) with an observed phenotype that includes smaller liver, smaller overall size and anemia. These observations suggest the critical role of SMRT and N-CoR in controlling important developmental pathways and indicate that, despite the high structural homology, they might not have overlapping cellular functions.

Data on breast cancer cell lines demonstrated that SMRT is important in regulating cell proliferation. Indeed, knockdown of both SMRT and N-CoR by siRNA increase cell proliferation rates in MCF7 cells (109) SMRT knockdown alone is sufficient to increase proliferation rates in breast cancer cells BT474 (110).

Co-regulators expression in breast cancer samples

Data on co-regulators expression in human breast samples are sparse and often contradictory. Many of the studies were conducted using small samples size and therefore with limited statistical power and non-uniform patients characteristics. This limits the interpretation of the data.

SRCs

SRC1 expression is increased in human breast tumors (53;111-115). There is compelling evidence that SRC1 positively correlates with HER2 status (53;111;114) while associations with other clinico-pathological variables are less well defined. Green et al. (116) showed a positive correlation with good prognostic factors such as ERα expression, low histological grade and small tumor size while Fleming et al. found a positive association with poor prognostic factors such as nodal positivity (53). Data on the association of SRC1 with patients outcome are controversial. Most of the studies show that patients with SRC1 overexpressing tumors are more likely to develop distant metastasis and that SRC1 is a strong predictor of shorter disease-free survival and overall survival (114;117), particularly in the HER2 positive population (111). However, in a recent study Green et al. demonstrated that tumors overexpressing SRC1 show longer overall survival and disease free interval (116).

There are very few studies investigating SRC2 in clinical samples probably due to the lack of reliable antibodies for this protein. One study reported a positive correlation of SRC2 with nodal status and ERβ expression (113).

AIB1 is amplified and over-expressed in breast cancer, although the frequencies reported in literature are variable (62;113;115;118-121). Most of the studies agree on the positive association of AIB1 with HER2 (119;122), while there is disagreement on the association with ER and tumor grade. Indeed, some studies show a positive correlation of AIB1 with ER and/or PR positivity and low tumor grade (114;116;120;121), while others show a negative association with those features (113;119). Associations have also been shown between AIB1 and cyclooxygenase- 2, phosphorylated extracellular signal- regulated kinase 1/2 (pERK1/2) (114), p53 and PEA3, MMP2, and MMP9 (74). Again, discrepancies exist regarding the prognostic role of AIB1. While one study demonstrated that patients whose tumors show elevated expression of AIB1 have significantly shorter disease-free and overall survival (118), Osborne et al. showed that high AIB1 expression in patients not receiving adjuvant endocrine therapy is associated with better prognosis and longer disease-free survival.

SMRT

To the best of our knowledge, to date there is only one study investigating SMRT expression by immunohistochemistry in breast cancer samples (116). This study shows that SMRT is an independent prognostic indicator of poor overall patient survival (OS) and disease free interval (DFI) and is significantly correlated with distant metastases and local recurrence (116).

Anti-estrogen therapy and tamoxifen resistance

The estrogen dependency of breast cancer is a unique feature of the disease that can be exploited to effectively control tumor growth. Indeed, current strategies for treatment of hormone-dependent breast cancer is to block estrogen action by:

a. Inhibiting estrogen from binding to ER, using SERM such as tamoxifen or pure antiestrogen agents such as fulvestrant (faslodex/ICI 182,780);

b. Preventing its synthesis (in postmenopausal patients), using an aromatase inhibitor.

Tamoxifen has been the primary line of therapy for ER positive breast cancer patients for nearly three decades and continues to be the choice of therapy for pre-menopausal patients (123). Data from adjuvant breast cancer trials have shown that 5 years of therapy with tamoxifen suppresses the recurrence of breast cancer

and reduces the incidence of contralateral second primary breast tumors by 50% (123). Tamoxifen has also beneficial effects as a chemopreventive agent reducing the chances of developing the disease by 50% in high-risk pre- and post-menopausal women (124).

Response to tamoxifen is rare in ER negative breast cancer; therefore ERα status (assessed by immunohistochemistry on breast specimens) is currently used to identify breast cancer patients who are likely to respond to tamoxifen. But nonetheless resistance occurs in 30 to 50% of treated ER positive breast cancer patients and development of tamoxifen resistance is a major clinical problem for long-term management of breast cancer. Loss of ER expression, increased metabolism of the drug, specific ER variants and/or ER mutations have been identified as potential mechanisms of resistance (125-127). However, loss of ER expression occurs in only a minority (15–20%) of resistant breast cancers and <1% of ER-positive tumors exhibit ER mutations. Furthermore, the majority of breast tumors seems to remain responsive to growth inhibition by pure anti-estrogens and other hormonal therapies (128).

Therefore other hypotheses have been investigated to explain the loss of tumor responsiveness to tamoxifen.

i. Numerous studies have identified molecular alterations in growth factor signaling and downstream pathways as potential drivers of endocrine resistance. In particular, early observations of reciprocal expression of ER and members of the epidermal growth factor receptor (EGFR) family such as EGFR and HER2 (129), the ability of growth factors to modulate tamoxifen sensitivity in vitro (130) and clinical data suggesting that patients with HER2- and EGFR-overexpressing tumors have a poorer outcome when treated with tamoxifen (131;132) have lead to the hypothesis that receptor tyrosine kinase expression and function can mediate endocrine resistance. Indeed, elevated expression of EGFR, HER2 and IGF-IR can elicit tamoxifen resistance (133-135), as well as the activation of components of their downstream signaling pathways, particularly the ERK/MAPK and PI3K pathways (136-138). In particular, overexpression of HER2 is one of the best-characterized mechanisms of endocrine resistance. Indeed several studies demonstrated that HER2/ER crosstalk have a central role in both *de novo* and acquired resistance to tamoxifen (139).

ii. It is very well known that ER can be phosphorylated and activated by multiple intracellular kinases (140). ER is phosphorylated at key residues (including serine 106/107, 118, 167, 305, and threonine 311) residing mainly in the AF-1 domain, by activated p42/44 MAPK, PI3K/AKT,

p90rsk, p21-activated kinase 1 (Pak1), protein kinase A, and p38 MAPK pathway in response to various cytokines and growth factors including ligands of EGFR or IGFR (141;142). ER phosphorylation has been shown to change ER pharmacology and can result in ligand-independent or tamoxifen-mediated activation of the receptor (143;144).

iii. Recent discoveries demonstrate an important role for co-activators and co-repressors in tamoxifen resistance. This findings will be discussed in detail below.

p 160 family and tamoxifen resistance

Preclinical and clinical data have linked SRC1 to tamoxifen resistance. In vitro experimental data, using a model of tamoxifen resistance, show that SRC1 expression is increased in resistant cells as compared to parental, suggesting that this co-activator may contribute to tamoxifen resistance (145). Moreover, multiple studies suggest that overexpression of SRC1 in breast cancer cell lines is able to enhance the agonist activity of tamoxifen and increase estrogen-stimulated expression of target genes (97;146;147). Clinical data show that expression of SRC1 is associated with resistance to endocrine treatment (53) and that SRC1 is a strong predictor of reduced disease-free survival (DFS) in patients receiving adjuvant tamoxifen treatment (114). However Berns et al. reported that SRC1 levels were lower in tumors from patients that did not respond to tamoxifen (148).

The role of AIB1 in tamoxifen resistance has been extensively analyzed. Scott et al., in a model of tamoxifen-resistant MCF7 cells, identified AIB1 as the most highly expressed co-activator (145) by real time RT-PCR; AIB1 was also similarly identified by microarray analysis (145) Like SRC1, also AIB1 enhances the agonist activity of tamoxifen in breast cancer cell lines (45;149). Using the MCF7/HER2–18 model, which is a derivative line of MCF7 cells that stably overexpresses HER2, Shou et al. demonstrated that culture of these cells under short-term tamoxifen treatment stimulates proliferation and increases the expression of estrogen-regulated genes nearly as well as estradiol itself. Both of these effects result from the HER2-driven phosphorylation of AIB1 (via extracellular signal-regulated kinase (ERK) -1/2), which enhances AIB1 co-activator function (150). It has also been shown that a balance between AIB1 and the transcriptional repressor PAX2 controls the estrogen-induced expression of HER2 in breast cancer cells. Tamoxifen resistance develops when AIB1 levels are high and PAX2 levels are low thus inducing high HER2 expression (151). Clinical data largely support the hypothesis that AIB1 over-expression is implicated in tamoxifen resistance, particularly in HER2 over-expressing tumors. Indeed three

independent studies demonstrated that patients whose tumors over-express both AIB1 and HER2 have the worse outcomes with tamoxifen therapy (114;152;153).

SMRT and tamoxifen resistance

Several studies have demonstrated that N-CoR and/or SMRT interact with ER in the presence of 4 hydroxytamoxifen (4HT) repressing the weak agonistic activity of this antiestrogen (96;97;99;154), and both 4HT and raloxifene have been shown to recruit N-CoR and SMRT to ER target genes (146;155;156). Emerging data have shown that knockdown of SMRT blocks tamoxifen-mediated inhibition of the expression of ER target genes and promotes cell growth in the presence of tamoxifen, indicating that co-repressor complexes are important players in tamoxifen-mediated transcriptional repression and anti-proliferative activity (147;157-159). Moreover, a mutant estrogen receptor (D351Y) has shown a reduced tamoxifen-dependent interaction with NCoR and SMRT and high tamoxifen-induced AF-1 activity, suggesting that potential interference with co-repressor binding to nuclear receptor might promote tamoxifen resistance (160). In addition, decreased levels of NCoR well correlated with the acquisition of tamoxifen resistance in a mouse model system of human breast cancer, suggesting that low N-CoR levels or activity could cause tamoxifen to act as an agonist rather than antagonist. (154).

Recent evidences suggest that SMRT activity is regulated by mitogenic signaling pathways. In the presence of tamoxifen, MEK inhibition enhances ER/SMRT interaction in MCF7 cells, suggesting that the MAPK signaling reduces SMRT recruitment to tamoxifen-bound ER (161). Additionally, phosphorylation of SMRT by Ras-MEKK1-MEK1 pathway results in a loss of affinity of the co-repressor for an assortment of receptor and non- receptor transcription factors, de-repression of previously repressed target genes, and redistribution of SMRT into a cytoplasmic/perinuclear location (104;105). Interestingly, a recent study showed that active SMRT forms homo-dimers, and that Erk2, a mitogen-activated protein (MAP) kinase, phosphorylation disrupts this SMRT self-dimerization in vitro and in vivo, therefore reducing its activity (162). This inhibition of co-repressor function by phosphorylation may contribute to the ability of EGFR and HER2 to counteract the antagonist properties of tamoxifen (154).

AIMS OF THE STUDY

Data on p160 family members and SMRT in human breast cancer samples and in endocrine treated patients are controversial or lacking altogether. In particular, there is limited information on the co- expression of multiple members of p160 family members in early breast cancer. Moreover, the role of these co-regulators in ER negative disease is poorly understood.

We hypothesized that

1. Co-operative overexpression of the different SRC family members would select for endocrine resistance in ER positive breast cancer patients treated with tamoxifen and combined expression levels of co-activators could improve selection of endocrine resistant breast cancers. We also hypothesized that HER2/SRC positive tumors would exhibit endocrine resistance. To test these hypotheses, we assessed SRC1, SRC2 and AIB1 in paraffin-embedded tissues from the Edinburgh breast unit, breast conservation series.

2. SMRT expression would select for endocrine resistance in ER positive cancers treated with tamoxifen. To test this hypothesis we assessed SMRT in paraffin-embedded tissue from the Tumor Bank and Data Network Core in the Lester and Sue Smith Breast Center at Baylor College of Medicine

MATERIALS AND METHODS

Tumor specimens and patients population

The Edinburgh Breast Conservation Series

The Edinburgh Breast Conservation series represents a fully-documented consecutive cohort of 1812 patients treated by breast conservation surgery, axillary node sampling or clearance, and whole breast radiotherapy at the Edinburgh Breast Unit between 1981 and 1998. Over this period a specialist multidisciplinary team including surgeons, radiologists, pathologists and oncologists managed patients. Eligible patients were those considered suitable for breast conserving therapy and were T1 or T2 (<3cm), N0 or N1 and M0 on conventional TNM staging. Postoperative breast radiotherapy was given over 4-5 weeks at a dose of 45Gy in 20-25 fractions. Data are available on adjuvant treatment, tumor size, estrogen receptor (ER), lymph node status and outcome with a minimum follow up of 9 years. Following ethical approval, tissue blocks were retrieved from all cases and sufficient material was available from 1686 cases for assembly into tissue microarrays (0.6-mm^2 cores in triplicate) (163). For the current study all patients from this group were stained for SRC1, SRC2 and SRC3/AIB1 (Table 1). These included tumors treated with adjuvant tamoxifen monotherapy (1102 cases), other hormonal therapy (92 cases) hormone and chemotherapy (149 cases) and chemotherapy alone (106 cases). In addition 197 cases received no adjuvant hormone or chemotherapy. At the end of the study, there were 297 breast cancer-specific deaths and 484 breast cancer relapses.

Tumor Bank and Data Network Core at the Lester and Sue Smith Breast Center

The Tumor Bank and Data Network Core in the Lester and Sue Smith Breast Center at Baylor College of Medicine is a prospectively assembled tumor bank. Tissue specimens were prepared from a cohort of 1424 frozen tumor specimens as previously described (164). Individual samples were fixed for 8 hours in 10% neutral buffered formalin and routinely processed to paraffin blocks. Samples were subsequently arrayed (12 samples/array; each core 5 mm in diameter). These uniformly prepared tissue samples have been already used to validate other prognostic and predictive factors in breast cancer including PR (165), ER (166) and IRS1 (167). The study population consisted of patients who were diagnosed between 1973 and 1998 with stage I and II primary breast cancer with no distant metastasis, treated with mastectomy or lumpectomy plus axillary dissection, with or without post-operative radiation therapy. Complete data on tumor size, number of nodes, receptors, S-phase fraction, ploidy and use and type of adjuvant therapy were available. Median follow-up was 84 months. For the current study all

patients were stained for SMRT. These included 695 patients who did not receive adjuvant therapy after primary treatment, 402 who received adjuvant tamoxifen monotherapy and 327 who received chemotherapy or a combination therapy.

Immunohistochemistry (IHC)

IHC was performed on tissue microarrays (TMAs) using a standard immunoperoxidase procedure with the following antibodies: SRC1 (128E7, Cell Signaling), SRC2/TIF2 (Clone 29, BD Biosciences), SRC3/AIB1 (Clone 34, BD Biosciences), SMRT (Clone 44, BD Biosciences). Antigen retrieval was performed by microwaving slides under pressure for 5min in TE buffer (1mM EDTA and 5mM Trisma base pH9; AIB1) or in citrate buffer pH6 (SRC1 and SRC2) or by heating in a pressure cooker for 10min in 0.1 M Tris-HCl buffer, pH 9.0 (SMRT). Nonspecific binding was blocked by incubating the tissue in Serum Free Block (DakoCytomation, Glostrup, Denmark) for 20 minutes. Endogenous peroxidase activity was quenched (all antibodies) and endogenous biotin blocked as previously described. Primary SRCs antibodies were applied at $4^{o}C$ overnight at the following concentrations: SRC1 (1:100), SRC2 (1:400) and AIB1 (1:50), while SMRT antibody was applied for 1 h at room temperature at the concentration of 1:300. EnVision (DakoCytomation) was used for signal amplification and positive staining was visualized using 3,3-diaminobenzidine tetrahydrochloride (DAB; Vector laboratories, CA, USA). Nuclei were counterstained with haematoxylin before mounting. SRC1 and SMRT staining were performed at the pathology core of Lester and Sue Smith Breast Center at Baylor College of Medicine, Houston, Texas, while staining for SRC2 and AIB1 were performed at the Edinburgh Cancer Research Centre, Edinburgh, UK, as specified in the Acknowledgment session.

Scoring of immunohistochemistry

For the SRCs study, nuclear SRC1 was scored blinded by two pathologists (myself and Carolina Gutierrez) according to the Allred score (168) and by an observer from the Edinburgh Cancer Research Centre, Edinburgh, UK with expertise in TMA analysis using a modified histoscore (169) There was a good correlation between the two score (data not shown); therefore nuclear SRC2 and AIB1 were scored at the Edinburgh Cancer Research Centre, according only to the modified histoscore. The histoscore was used for the subsequent statistical analysis of SRCs.

For the SMRT study, immunostained slides were evaluated for both nuclear and cytoplasmic SMRT. Nuclear SMRT was scored blinded according to the Allred score by two pathologists (myself and Carolina Gutierrez) (168). Briefly, each entire core was evaluated by light microscopy. First, a proportion score was assigned, which represents the estimated proportion of positive-staining tumor cells (0, none; 1, <1/100; 2, 1/100 to 1/10; 3, 1/10 to 1/3; 4, 1/3 to 2/3; and 5 > 2/3). Next, an intensity score was assigned, which represents the average intensity of positive tumor cells (0, none; 1, weak; 2, intermediate; 3, strong). The proportion and intensity scores were then added to obtain a total score, which ranged from 0 to 8. Cytoplasmic SMRT was evaluated based on the intensity of the staining according to a score that ranges from 0 to 3 (0, none; 1, weak; 2, intermediate; 3, strong)(168).

Statistical analysis

SRCs status was categorised as high (above upper quartile) or low (below upper quartile). Nuclear SMRT status was categorized in quartiles (1^{st} ≤4; 2^{nd} >4 and ≤6; 3^{rd} =7; 4^{th} =8) while cytoplasmic SMRT status was dichotomized in negative (=0) and positive (>0).

Spearman rank tests were conducted to test the associations between each co-regulator and molecular or clinical markers.

Univariate analysis on recurrence-free survival (RFS), distant relapse-free survival (DRFS) and overall survival (OS) was carried out using the Kaplan-Meier method and compared using the log-rank test.

The prognostic and predictive significance of co-regulators was analyzed by Cox proportional hazards regression models. All variables of interest were entered into multivariate Cox regression models and model-building proceeded using stepwise selection.

Clinico-pathological variables were categorized according to standard cut-offs.

Data analysis for SRCs study were conducted at the Edinburgh Cancer Research Centre, while data analysis for SMRT study were conducted at Baylor College of Medicine (as specified in the Acknowledgment session)

RESULTS

Patients and tumors characteristics

The Edinburgh Breast Conservation Series

Clinical and pathological data for the patients are shown in Table 1. A total of 1686 cases were studied, including 1277 patients with ER positive tumors and 316 with ER negative tumors. The majority of the patients were older than 50 years of age, with small (<2 cm), node negative tumors. PR was expressed in 78% of the ER positive tumors. Ten percent of all tumors were HER2 positive.

Table 1: Edinburgh Breast Conservation series: Patient clinical and pathological characteristics by subgroup

		All cases (1686)	All ER+ve (1277)	ER-ve (316)
Age (y)	≤50	529	392	122
	>50	1157	885	194
	Missing	0	0	0
Grade	1	427	373	25
	2	745	605	99
	3	472	280	184
	Missing	42	19	8
Node status	Negative	1217	921	214
	1-3	386	304	102
	4-9	59	37	0
	10+	23	14	0

	Missing	1	1	0
Size (cm)	≤2	1227	947	198
	>2	377	264	108
	Missing	82	65	10
ER	ER-ve	316	NA	NA
≤2	ER+ve	1277	NA	NA
	Missing	93	NA	NA
PR	PR-ve	272	135	127
≤2	PR+ve	1316	1114	182
	Missing	98	28	7
HER2	HER2-ve	1189	961	194
	HER2+ve	175	115	58
	Missing	322	201	64
SRC1	SRC1-ve	984	772	192
	SRC1+ve	367	310	46
	Missing	335	195	78
SRC2	SRC2-ve	1028	798	187

	SRC2+ve	342	285	47
	Missing	316	194	82
SRC3	SRC3-ve	1125	873	222
	SRC3+ve	380	300	73
	Missing	181	104	21

Tumor Bank and Data Network Core at the Lester and Sue Smith Breast Center

The distributions of the patient's clinical characteristics are presented in Table 2**.** A total of 866 patients were studied including 765 patients with ER positive tumors and 101 with ER negative tumors. The majority of patients were older than 50 years of age. We included in the analyses only patients with tumors less than 5 cm in diameter, the majority of which was node negative. About 70% of ER positive tumors were also PR positive. Approximately 64% of tumors were of low to intermediate S-phase, and nearly 60% were aneuploid. For this population we also stratified patients based on treatment: 330 patients were treated with adjuvant tamoxifen monotherapy and 536 patients received no adjuvant therapy after their primary treatment.

Table 2: Tumor Bank and Data Network at Lester and Sue Smith Breast Center: Patient clinical and pathological characteristics by subgroup

		All		Untreated				Treated
		(866)		ER+ (435)		ER- (101)		ER+ (330)
Age(y),	≤50	162(18.7)		91(20.9)		41(40.6)		30(9.1)
	>50	704(81.3)		344(79.1)		60(59.4)		300(90.9)
Size (cm)	<2	351(41.1)		191(44.5)		30(30.6)		130(39.6)
	>2-5	504(58.9)		238(55.5)		68(69.4)		198(60.4)
	Missing	11						
Node status	Negative	629(72.6)		360(82.8)		89(88.1)		180(54.5)
	Positive							
	1-3	143(16.5)		50(11.5)		9(8.9)		84(25.5)

	>3	94(10.9)		25(5.7)		3(3.0)		66(20.0)
S phase	Low (0 to <6%)	230(32.5)		131(36.8)		11(13.6)		88(32.5)
	Intermediate (≥6 to ≤10%)	219(30.9)		116(32.6)		10(12.3)		93(34.3)
	High (>10%)	259(36.6)		109(30.6)		60(74.1)		90(33.2)
	Missing	158						
Ploidy	Diploid	288(38.8)		162(43.9)		17(20.0)		109(37.7)
	Aneuploid	455(61.2)		207(56.1)		68(80.0)		180(62.3)
	Missing	123						
PR (fmol/mg)	Negative (<5)	317(37.7)		137(32.7)		85(86.7)		95(29.4)
	Positive (≥5)	523(62.3)		282(67.3)		13(13.3)		228(70.6)
	Missing	26						
Nuclear SMRT	1st quartile (<=4)	237(27.4)		131(30.1)		28(27.7)		78(23.6)
	2nd quartile (>4,<=6)	256(29.6)		132(30.3)		25(24.8)		99(30.0)
	3rd quartile (=7)	181(20.9)		85(19.5)		26(25.7)		70(21.2)
	4th quartile (=8)	192(22.2)		87(20.0)		22(21.8)		83(25.2)
Cytoplasm SMRT	Negative (=0)	581(67.1)		289(66.4)		68(67.3)		224(67.9)
	Positive (>0)	285(32.9)		146(33.6)		33(32.7)		106(32.1)
Follow-up time (mo)		86		86		83		85

Co-regulators expression

SRCs

SRC1, SRC2 and AIB1 expression was confined to the nuclei of invasive tumor cells, with no staining in normal breast epithelial cells (Figure 1). 21.8% of cases

were SRC1 positive, while 58.4% were negative; 20.3% were SRC2 positive, while 61% were negative; 22.5% were AIB1 positive, while 66.7% were negative (Table 1). The frequency histogram for expression of each of the SRC proteins varied. SRC1 expression ranged from a histoscore of 0-245, with a significant group of cases having uniform low level (1+) staining. SRC2 staining exhibited the narrowest range with a maximum histoscore of 196. Staining for AIB1 ranged from 0-290 and exhibited the highest median staining.

SMRT

SMRT was expressed in the nuclei and in the cytoplasm of invasive tumor cells with a low nuclear staining present in the normal breast epithelial cells, which served as internal positive control (Figure 2). Fifty-seven percent of the tumors exhibited low nuclear SMRT expression (1st and 2nd quartile) while the highest SMRT expression (4th quartile) was observed in 22.2 % of the specimens (Table 2). The majority of tumors (67.1%) were negative for cytoplasmic SMRT expression (Table 2).

Correlation with clinico-pathological characteristics

SRCs

We assessed the correlation between SRC1, SRC2 and AIB1 expression with various clinico-pathological parameters (table 3). SRC1 expression showed a significant, albeit small, positive correlation with ER (r = 0.198; $P = 4x10^{-13}$) and PR expression (r = 0.132 $P = 1.5x10^{-6}$). SRC2 and AIB1 were significantly associated to each other (r = 0.136; $P = 1.6x10^{-6}$) and AIB1 was positively and significantly associated with grade (r = 0.213; P = 0.0000002) and inversely associated with PR expression (r = -0.278; $P = 1.6x10^{-27}$) and with Ki67 expression (r = -0.103; $P = 8x10^{-5}$).

Table 3: Correlations between SRCs and clinico-pathological characteristics

	SRC1	SRC2	SRC3/AIB1
Grade	-0.133 p=0.002	NS	0.213 **p=0.0000002**
Nodal Status (+ve vs –ve)	NS	NS	NS
Size	NS	0.056 p=0.043	0.074 p=0.005
ER	0.198 **p=4x10^{-13}**	0.07 p=0.011	NS
PR	0.132	NS	-0.278

	$p=1.5x10^{-6}$		**$p=1.6x10^{-27}$**
HER2	NS	NS	NS
Ki67	-0.076 p=0.006	NS	-0.103 **$p=8x10^{-5}$**
SRC1	NA	NS	NS
SRC2	NS	NA	0.136 **$p=1.6x10^{-6}$**
SRC3/AIB1	NS	0.136 **$p=1.6x10^{-6}$**	NA

SMRT

The results of the correlation analysis of SMRT expression with clinico-pathological parameters are presented in Table 4. Nuclear and cytoplasmic SMRT expressions were modestly correlated with each other (r = 0.150; $P < 0.0001$). There was a small negative correlation between nuclear SMRT and lymph node involvement (r = -0.073; P = 0.030) whereas there were small positive correlations observed between nuclear or cytoplasmic SMRT and S-phase (r = 0.087; P = 0.020 and r = 0.131; P = 0.0005, respectively). We observed small, albeit significant, correlations between nuclear or cytoplasmic SMRT and ER (r = 0.069; P = 0.043 and r = 0.089; P = 0.009, respectively). However we found no significant correlations between SMRT, either cytoplasmic or nuclear, and age, PR status or tumor size.

Table 4: Correlations between SMRT and clinico-pathological characteristics

	All (n=866)	
	Nuclear	Cytoplasm
	Correlation (P)[†]	Correlation (P)[†]
Nuclear SMRT[‡]	1	0.150(<0.0001)
Cytoplasmic SMRT[‡]	0.150(<0.0001)	1
Age	-0.025(0.455)	-0.041(0.223)
ER	0.069(0.043)	0.089(0.009)
PR	0.048(0.167)	0.040(0.244)

Tumor Size	-0.020(0.563)	0.040(0.239)
Nodes	-0.073(0.030)	-0.007(0.840)
S Phase	0.087(0.020)	0.131(0.0005)

†: Spearman rank correlation.

‡: Correlation between nuclear and cytoplasm

Univariate analysis of SRCs

SRCs expression in the overall population

In all patients (both ER positive and ER negative), only AIB1 was significantly associated with RFS, DRFS and OS (Table 5A).

SRCs expression in ER positive patients

When divided according to ER status, we found no significant association between any individual SRC family member expression and outcome in patients that were ER positive and treated with either any hormone therapy or tamoxifen (data not shown). There was only a non-significant trend (after correction for multiple testing) for reduced DRFS in patients with ER positive tumors with AIB1 overexpression treated with tamoxifen (HR: 1.52, 95%CI 1.04-2.23; p = 0.033). Therefore none of the member of SRC family alone represents a predictive marker of endocrine resistance.

SRCs expression in ER negative patients

High expression of SRC1 and AIB1 were associated with reduced RFS, DRFS and OS in ER negative breast cancers (Table 5B), although only AIB1 was significantly associated with reduced DRFS in ER negative breast cancers following correction for multiple testing. Combining overexpression of either SRC1 or AIB1 confirmed the negative prognostic impact with respect to RFS and DRFS in univariate Cox regression analyses (Table 5B; Figure 3A-C). Overexpression of both SRC1 and AIB1 was a rare event, seen in only 6 cases of ER negative breast cancer (data not shown).

Overexpression of two or more SRC family members

When ER positive cases were stratified according to the number of overexpressed SRC family members (ER+ve/no SRC high expression, ER+ve/ high expression of one SRC family member, or ER+ve/high expression of two or more SRC family members), there appeared to be a significant relationship between SRC expression and outcome. Tumors that overexpressed only one SRC family member (344/882;

45%) were associated with improved RFS (p=0.005: Figure 4A) and DRFS (p=0.004: Figure 4B) compared to tumors that did not overexpress any SRC family member (400/882; 45%) and tumors with high expression of two or more family members. Conversely, those tumors with high expression of two or more SRC family members (138/882; 16%) were associated with decreased DRFS (p = 0.004) with a 2.2-fold (95% CI 1.4-3.6, p = 0.004) increase risk of distant relapse when compared to patients that expressed only one SRC family member. No significant association was observed between overexpression of the co-activators and OS (data not shown). Furthermore, no significant association was observed when other prognostic factors such as nodal status, grade, size and HER2 status was included in the multivariate analysis (data not shown).

Table 5: Univariate Analysis of SRCs

	RFS	DRFS	OS
A: ALL PATIENTS			
SRC1	0.99 (0.79-1.24)	0.95 (0.73-1.25)	0.95 (0.70-1.28)
SRC2	0.91 (0.72-1.16)	0.95 (0.72-1.26)	1.00 (0.74-1.35)
AIB1	1.30 (1.05-1.60) 0.015	1.53 (1.21-1.93) **0.0004**	1.50 (1.16-1.94) **0.002**
B: ER-ve cases			
SRC1	1.81 (1.12-2.93) 0.015	1.79 (1.05-3.06) 0.032	1.55 (0.87-2.76)
SRC2	0.82 (0.45-1.50)	0.85 (0.44-1.62)	0.89 (0.45-1.76)
AIB1/SRC3	1.50 (0.99-2.28) 0.055	1.86 (1.19-2.90) **0.006**	1.49 (0.91-2.41)
SRC1/SRC3 vs not	1.91 (1.25-3.10) **0.004**	2.25 (1.36-3.72) **0.002**	1.87 (1.09-3.19) 0.023

AIB1 and HER2 expression as a combined biological marker of poor outcome.

The subgroup of HER2 positive tumors with high levels of AIB1 has previously been identified as having poor outcome on endocrine therapy (153). We observed a significant reduction in RFS, DRFS and OS in both HER2 and HER2/AIB1 positive tumors (Table 6). Tumors with high expression of HER2 alone exhibited

a 40-60% increased risk of relapse and death, whilst tumors with high expression of both markers exhibited increased in relapse risk ranging from 121-216% greater than tumors without either AIB1 or HER2 overexpression. Tumors that overexpressed AIB1 alone were associated with between 10-40% increase risk of relapse or death (Table 6; Figure 5A-C).

Whilst the group of HER2/AIB1 overexpressing tumors represents a small minority of cases (3% of the total population) it represents a significant subgroup of HER2 positive disease (42/168 HER2 positive cases, 25%). Additionally, despite the small number of patients in this group, multivariate analysis, including nodal status, grade, size, menopausal status, ER and PR, suggests that stratification by HER2/AIB1 status remained an independent prognostic variable for DRFS within this cohort (data not shown).

Table 6: Exploratory multivariate regression analysis of AIB1/HER2 groups:

Factor	RFS	DRFS	OS
AIB1/HER2 Groups	p=0.002	p=0.0001	p=0.002
Nodal status	p<0.00001	p<0.00001	p<0.00001
Size	p=0.0001	p=0.0002	p=0.00008
Grade	p=0.013	p=0.024	p=0.001
Menopausal status	NS	NS	NS
ER	p=0.034	p=0.024	p=0.016
PgR	NS	NS	NS

Univariate analysis of SMRT

SMRT expression in the overall population

Considering the entire population of untreated patients (both ER-positive and ER-negative) there was a strong and significant positive correlation between low nuclear SMRT expression (1st quartile, Allred score < or = 4) and longer RFS (P = 0.007; Figure 6A) while no correlation was seen between nuclear SMRT expression and OS (P = 0.603, Figure 6B).

SMRT expression in ER positive patients

When dived according to ER status, untreated patients with ER-positive tumors, which expressed low nuclear SMRT expression (1st quartile) had a significantly better RFS (P = 0.01 Figure 7A) compared to those with greater nuclear SMRT

expression, while there was no significant difference in OS (Fig. 7C). Moreover, we did not observe significant differences in either RFS or OS between quartiles of SMRT expression among tamoxifen-treated patients (Figs. 7B & D). Therefore, in ER positive population, low nuclear SMRT represents a marker of good prognosis, being associated with a relative delay in tumor recurrence in untreated patients. However, nuclear SMRT expression does not represent a predictive marker of response to tamoxifen treatment.

No differences in RFS or OS were found between positive and negative cytoplasmic SMRT in the untreated population (P = 0.783 and P = 0.957, respectively; data not shown).

SMRT expression in ER negative patients

In the relatively small number of patients with ER-negative tumors (n=101), patients with higher SMRT expression showed a trend towards decreasing RFS (P = 0.079; Figure 8A), while no difference was found in OS (Figure 8B).

Multivariate regression analysis:

SRCs

The multivariate regression analysis of SRCs study is shown in Table 7,

In a multivariate regression analysis of all cases (both ER positive and ER negative) SRC1 expression was significantly associated with reduced DRFS (HR = 1.40; 95% CI, 1.00-1.96; P = 0.048) as was HER2 expression, nodal status, size.

In ER positive disease, no SRC was associated with RFS, DRFS or OS. In this analysis nodal status, grade, size and HER2 levels were significant.

In ER negative disease, expression of either SRC1 or AIB1 was associated with reduced RFS, DRFS, and OS (HR = 1.94; 95% CI, 1.19-3.17; P = 0.008 and HR = 2.27; 95% CI, 1.33-3.88; P = 0.003 and HR = 1.90; 95% CI, 1.07-3.38; P = 0.028, respectively) in multivariate analysis; only nodal status was also significant in this analysis (probably due to the small sample size).

Table 7: Multivariate regression analysis in SRCs study:

	ER-ve	ER+	All	
Relapse Free Survival				
Nodal Status	3.15 (1.87-5.31) p<0.0001	1.72 (1.29-2.30) p<0.0001	2.01 (1.56-2.58) p<0.0001	

Grade	NS	1.57 (1.06-2.33) p=0.044	1.66 (1.16-2.38) p=0.019	
Size	NS	2.02 (1.50-2.71) p<0.0001	1.58 (1.22-2.04) p=0.001	
Menopausal status	NS	NS	NS	
Age	NS	NS	NS	
HER2	NS	1.50 (1.03-2.20) p=0.036	1.48 (1.09-2.02) p=0.013	
PgR	NS	NS	NS	
ER	NA	NA	NS	
SRC1/SRC3	1.94 (1.19-3.17) p=0.008	NA	NA	
SRC1	NA	NS	NS	
SRC2	NA	NS	NS	
SRC3	NA	NS	NS	
Distant Disease Free Survival				
Nodal Status	3.31 (1.88-5.81) p<0.0001	2.15 (1.52-3.03) p<0.0001	2.37 (1.77-3.18) p<0.0001	
Grade	NS	NS	NS	
Size	NS	1.96 (1.38-2.79) p=0.0002	1.64 (1.21-2.22) p=0.001	
Menopausal status	NS	NS	NS	
Age	NS	NS	NS	
HER2	NS	1.77 (1.15-2.72) p=0.009	1.63 (1.15-2.32) p=0.006	
PgR	NS	NS	NS	
ER	NA	NA	NS	
SRC1/SRC3	2.27 (1.33-3.88) p=0.003	NA	NA	
SRC1	NA	NS	1.40 (1.00-1.96)	

			p=0.048	
SRC2	NA	NS	NS	
SRC3	NA	NS	NS	
Overall Survival				
Nodal Status	3.77 (2.07-6.90) p<0.0001	2.18 (1.49-3.18) p<0.0001	2.47 (1.79-3.41) p<0.0001	
Grade	NS	2.35 (1.34-4.13) p=0.009	2.25 (1.34-3.79) p=0.002	
Size	NS	2.20 (1.50-3.23) p0.0001	1.78 (1.29-2.46) p=0.001	
Menopausal status	NS	NS	NS	
Age	NS	NS	NS	
HER2	NS	1.66 (1.03-2.67) p=0.039	1.48 (1.01-2.18) p=0.047	
PgR	NS	NS	NS	
ER	NA	NA	0.67 (0.45-0.99) p=0.046	
SRC1/SRC3	1.90 (1.07-3.38) p=0.028	NA	NA	
SRC1	NA	NS	1.44 (1.00-2.07) p=0.05	
SRC2	NA	NS	NS	
SRC3	NA	NS	NS	

SMRT

The prognostic and predictive effects of clinico-pathological variables (nuclear SMRT, cytoplasmic SMRT, age, PR, nodes) on RFS in untreated ER positive and tamoxifen-treated patients are shown in Table 8. In multivariate analysis of RFS for untreated patients, nuclear SMRT expression was significantly associated with RFS (HR = 1.73; 95% CI, 0.98-3.06; *P* = 0.032). Cytoplasmic SMRT was not

associated with RFS. For tamoxifen-treated patients, there was no significant association between either cytoplasmic or nuclear SMRT and RFS.

Among patients treated with tamoxifen, those with an age at diagnosis of 50 years or younger had a worse RFS than patients older than 50 years old (HR = 3.33; 95% CI, 1.84-6.02, *P* <0.0001). Negative PR status also was associated with earlier recurrence for tamoxifen-treated patients (HR = 1.66; 95% CI, 1.07-2.59, *P*= 0.024). For both untreated and tamoxifen-treated patients, node status (three or more positive nodes) was a significant indicator of recurrence (HR = 3.27; 95% CI, 1.82-5.87, *P* <0.0001 and HR = 3.96; 95% CI, 2.38-6.60, *P* <0.0001, respectively).

Table 8: Multivariate regression analysis of SMRT study in ER positive patients.

RFS - Untreated (n=435)

Variable	HR	95% CI	P
Nuclear			0.032
1st quartile (<=4)	1.00	–	
2nd quartile (>4,<=6)	2.05	1.25-3.37	
3rd quartile (=7)	1.96	1.12-3.40	
4th quartile (=8)	1.73	0.98-3.06	
Cytoplasm			0.286
Negative (=0)	1.23	0.84-1.81	
Positive (>0)	1.00	–	
Node			0.0003
Node Negative	1.00	–	
1-3	1.49	0.90-2.47	
>3	3.27	1.82-5.87	

RFS - Treated (n=323†)

Variable	HR	95% CI	P
Nuclear			0.132
1st quartile (<=4)	1.00	–	
2nd quartile (>4,<=6)	1.04	0.59-1.85	
3rd quartile (=7)	0.59	0.29-1.22	
4th quartile (=8)	1.39	0.75-2.59	
Cytoplasm			0.179
Negative (=0)	1.40	0.86-2.30	
Positive (>0)	1.00	–	
Age			<0.0001
<=50	3.33	1.84-6.02	
>50	1.00	–	
PR			0.024
Negative	1.66	1.07-2.59	
Positive	1.00	–	
Node			<0.0001
Node Negative	1.00	–	
1-3	1.17	0.66-2.09	
>3	3.96	2.38-6.60	

†: 7/330 tamoxifen-treated patients did not have PR data.

DISCUSSION

Breast cancer has been paradigmatic of how bench-to-bedside breakthroughs can ultimately result in life-saving treatment strategies. The recognition that many breast cancers are hormone-dependent (5) led to identification of estradiol and its receptor, ER as the main driver of breast tumor progression. Afterward, the evidence that only one third of women with breast cancer responded to any form of endocrine ablative therapy, raised the question of whether ER expression could predict therapy response. Jensen EV et al first reported in 1971 that ER-rich breast cancers were more likely to respond to endocrine ablation (170). Therefore ER became the first known predictive marker in the history of oncology. In the early 1970s tamoxifen was developed. The strong biological evidence that tamoxifen blocks the binding of estradiol to ER in human breast and rat mammary tumors and prevents the induction and growth of ER positive carcinogen-induced rat mammary carcinomas, (171-174) set the basis for the development of endocrine therapy, the first recognized target therapy for human cancer. It is now known that five years of adjuvant tamoxifen is unable to provide any benefit for patients with ER negative tumors, while it result in a 50% decrease in recurrences and a decrease in mortality 15 years after diagnosis in ER positive patients (175). Therefore tamoxifen is paradigmatic of how a target agent can be successfully used only in a population harboring the target.

Nevertheless, approximately 50% of ER-positive breast cancers are innately resistant to endocrine therapies and almost all patients who do respond will eventually become unresponsive despite the continued presence of both the anti-estrogen and a functional receptor. In order to build advanced personalized treatment strategies and further reduce breast cancer mortality, it is necessary to identify patients at higher risk of recurrence or that would benefit from specific endocrine therapies.

To identify such new potential prognostic and predictive markers it is important to understand the biology of breast cancer and the molecular mechanisms underlying endocrine resistance. It is now clear that tamoxifen resistance results from an imbalance between anti-estrogens agonist and antagonist actions and there is strong biological evidence that co-regulators are involved in breast cancer progression and in tamoxifen resistance. However the question of what are the clinical implications of these findings remains open.

We tried to answer this question by analyzing levels of expression of ER co-regulators in human breast cancer samples with the aim of determining whether

these could improve breast cancer classification, assessment of prognosis and/or prediction of response to endocrine therapy in patients with early breast cancer.

In our study, the largest to date of this kind, expression levels of SRC1, SRC2, AIB1 and SMRT were determined in two large and well characterized cohorts of breast tumor samples. In particular the Tumor Bank and Data Network Core at the Lester and Sue Smith Breast Center is a unique tumor bank. This bank includes tumor samples from patients diagnosed between 1973 and 1998, thus comprising patients who did not receive any adjuvant therapy after surgery. The presence of an untreated group of patients as well as of a tamoxifen only treated population in this bank allowed us to better discriminate between the prognostic (natural progression) and predictive (response to drug) effect of SMRT expression without the confounding of other treatments (e.g. chemotherapy).

Immunohistochemistry was used to explore protein expression in breast cancer TMAs, which were then analyzed by either histoscore or Allred score methods (168). IHC is an easy, inexpensive, safe technique that can be applicable to a wide variety of samples (e.g. cytologic preparations, frozen tissue sections, fixed archival tissue sections, etc). Moreover it is very sensitive and specific in the identification of rare positive tumor cells under direct microscopic visualization. TMAs have been widely established as reliable and enable high- throughput simultaneous analysis of a large number of tumor samples (176;177). Both the histoscore and the Allred score have been previously demonstrated for ER to be reliable and produce equivalent results to other immunohistochemical scoring methods and biochemical methods, such as ligand-binding assay (166;178). Additionally, in a preliminary analysis, we found a good agreement between the two scoring methods (data not shown).

In this study we found protein expression of SRC1, SRC2 and AIB1 to be localized in the nuclei. Conversely, SMRT staining was found to be localized both in the nuclei and in the cytoplasms of invasive breast cancer cells. Regarding AIB1, some studies reported this co-activator as being predominantly nuclear (112;113), while others reported cytoplasmic staining (115;116;120). Indeed, List et al. suggested a trend that, with increased progression of breast cancer, AIB1 localization becomes more nuclear than cytoplasmic (115). This was not shown in the current study where we found an exclusively nuclear staining for AIB1. This discrepancies between the studies could be due to the different reagents used. Indeed, our study was carried out with an AIB1 antibody produced by BD Biosciences (BD) while Green et al used the AIB1 antibody from Santa Cruz Biotechnology (SC). In order to understand the differences seen in the AIB1 protein expression patterns on IHC with these two AIB1 antibodies, Balmer NN et al. performed a Western Blot analysis on protein extract from cells transfected

with an expression vector encoding human AIB1 *vs* untransfected cells (179). They demonstrated that the AIB1 (BD) antibody detected a single protein band migrating according to the appropriate molecular weight (160 kDa) of the AIB1 protein, while the SC antibody, in addition to a band migrating at the appropriate molecular weight, more strongly detected an additional non-specific band. This result may suggest that the cytoplasmic staining observed when this antibody is used for IHC could result from cross reactivity to a non-specific protein. We used a different antibody (BD) compared to Green et al (SC) also for SMRT staining (116). Our antibody detected SMRT in both nuclei and cytoplasms while the SC antibody detected SMRT only in the nuclei (116). Again, different specificity of these antibodies might explain the differences in the staining pattern. However, our finding is not surprising and the cytoplasmic staining seen in this study may have functional implications. Many other transcription factors, such as BRCA1, are found in the cytoplasm reportedly due to inactivation and mislocalisation (180). Additionally, Hong SH et al. show that SMRT sub-cellular distribution can be changed from an exclusively nuclear compartment to a more perinuclear and cytoplasmic distribution due to MEK-1 signaling (105).

Here we analyzed the associations of SRC1, SRC2, AIB1 and SMRT with various clinical and pathological parameters, as well as with the expression levels of nuclear receptors ER and PR. In agreement with a previous study (116), we demonstrated a significant correlation between AIB1 and SRC2 expression. However, in contrast to Green et al. we found no correlation between AIB1 or SRC2 and SRC1. Our sample size was large enough to perform logistic regression analyses, which may provide more robust estimate of interactions between genes compared to other studies. We found significant correlations between SRC1, PR and ER expression with no significant correlation between AIB1 and ER as well as SRC2 and either ER or PR. While the association between SRC1 and ER/PR is in agreement with Green et al, these authors, in contrast to our study, also identified a borderline association between ER and AIB1 expression. Moreover in the current study AIB1 expression was inversely related to expression of PR, again in contrast to Green et al., which found no correlation. Green et al. also showed no correlation between SMRT and ER or PR, while in the current study we found a weak, but significant, correlation between both nuclear and cytoplasmic SMRT and ER. There seems to be no consensus in the literature as to the relationships between steroid receptor expression and that of co-regulators. However others have previously suggested an independence of co-regulators and ER (111;113;181) or PR expression (113;182). Overexpression of AIB1 has been previously associated with high grade and lymph node positive breast cancer (69;116) and we confirm the relationship between overexpression of AIB1 and grade, but not nodal status, in the current study. We also observed a weak relationships between tumor size and AIB1 expression, which contrasts with

previous data (Green et al) and between S-phase fraction and nuclear SMRT. Neither our study nor that of Green et al. showed any relationship between SRCs expression and HER2. Differences in the results between the studies may be explained by the study of different cohorts, small numbers of tumors and technical differences in antibodies and staining, but it should also be noted that in some of these studies the associations are relatively weak and thus may be difficult to replicate. However, the considerably large sample size and the semi-quantitative assessment of protein expression performed in the current study give great reliability to our results.

Previous data from Green et al (116) suggested that, in an untreated breast cancer population, overexpression of SRC1 is associated with improved outcome; SCR2 and SMRT perform as poor prognostic factors, whilst AIB1 has no prognostic impact. In the current study, among co-activators, we found only AIB1 to be associated with poor outcome when examined in all patients (both ER positive and ER negative and treated/untreated). In agreement with Green et al., we found that nuclear SMRT was associated with a poor recurrence free survival when examined in untreated patients (both ER positive and ER negative subgroup).

When patients were divided based on ER status, we found the steroid receptor co-regulators AIB1, SRC1 and SMRT to be associated with a significant negative prognostic impact in ER negative breast cancer. Approximately a third of patients with ER negative disease exhibited high levels of AIB1 or SRC1 expression associated with a doubling in the risk of both local and distant recurrence and ultimately death in both univariate and multivariate regression analyses. Moreover, patients with ER negative breast cancers and high levels of nuclear SMRT showed a trend toward increased risk of local recurrence. Although not significant, probably due to the small sample size, it also looks like nuclear SMRT acts in a "dose-dependent" manner with a better survival showed by patients within the 1st quartile of nuclear SMRT expression and an intermediate risks of local recurrence showed by patients within 2nd and 3rd quartile. To the best of our knowledge, this is the first evidence implicating these co-regulators with prognosis of ER negative breast cancers. Previous studies, including that by Green et al (116) did not perform analyses separately for ER positive and ER negative disease. There is some prior evidence that overexpression of AIB1 in ER negative breast cancer is associated with poor patient outcome (183), however, in general, clinical evidence for the impact of overexpression of nuclear co-regulators in ER negative disease is relatively sparse. Although at first thought the impact of ER co-regulators might look somewhat perplexing, there are an increasing number of preclinical studies that show critical roles for co-regulators in ER-independent breast cancer. For example, Louie et al have shown that AIB1 increases expression of E2F1-induced genes such as Cyclin E and Cdk2, which promote cell proliferation (66). In

addition, AIB1 may promote activation of AP1 and NFkB signaling independently of ER (184;185). Recent in vivo data, using various animal models, shows that AIB1 interacts with both ER dependent and independent pathways in the promotion of early oncogenesis (186). There is also growing evidence that SRC1 plays a critical role in metastasis in ER-independent processes. Disruption of the SRC1 gene in mouse models decreases breast cancer metastasis, and although the detailed mechanisms have yet to be discovered, candidate pathways include SRC1/PEA3-mediated induction of Twist, SRC1 role in Ets-2-mediated HER2 expression, and finally activating CSF-1 expression for macrophage recruitment to the tumor site (57;58;74). Similarly, SMRT might also play a role in ER-independent processes. Indeed, while SMRT have never been assigned a direct role in regulating the cell cycle, its levels have been reported to fluctuate during mitosis (81). Several recent reports have identified a novel role for HDAC3, a deacetylase directly regulated by SMRT, in cell cycle regulation. In terms of transcriptional regulation, HDAC3 has been shown to repress several critical cell cycle regulators such as the E3 ubiquitin ligase Skp2 (187;188) and several Cdk inhibitors (189). Since SMRT is critical for HDAC3 deacetylase activity, it could be that SMRT may also be important for these activities and thus critical for normal cell cycle progression. Further in vitro and in vivo work would need to be carried out to decipher the detailed role of co-regulators in ER-independent tumorigenesis and progression. However, our clinical observations would suggest that SRC1, AIB1 and nuclear SMRT are potentially important in progression of ER negative breast cancers.

In the ER positive population, we found no significant association between any individual SRC family member expression and outcome in patients that were treated with either any hormone therapy or tamoxifen. There was only a non-significant (after correction for multiple testing) trend for reduced DRFS in patients with ER positive tumors with AIB1 overexpression treated with tamoxifen. Osborne et al. previously found that, in patients known to have ER-positive primary breast cancer who were treated by surgery followed by adjuvant tamoxifen therapy, high levels of AIB1 were associated with poor DFS in both univariate and multivariable analyses. However, there are considerable differences between their study and the current one. They used Western blot analysis, while we used IHC; they looked at a much smaller sample size (n=316 vs n=1686) and their cohort included only patients with positive lymph nodes. To our knowledge this is the first study analyzing SMRT expression by IHC in patients with primary breast cancer treated with tamoxifen monotherapy. Biologic evidence suggests a mechanism whereby SMRT might modulate the estrogen agonist or antagonist properties of tamoxifen or other SERMs to influence their anti-tumor activity in patients (96;97;99;147;154;157-159). Therefore it would be expected for SMRT expression to predict tamoxifen response in breast cancer patients. However, we

found that, in the ER positive population, low levels of nuclear SMRT were associated with improved recurrence free survival in untreated patients, both at univariate and multivariate analysis, therefore representing a good prognostic marker, but we did not see any difference in either recurrence free survival or overall survival in tamoxifen treated patients. Hence nuclear SMRT does not represent a predictive marker of tamoxifen response. Additonally, cytoplasmic SMRT did not correlate with outcome. Previous studies have already failed to detect changes in SMRT expression for tamoxifen-resistant breast tumors or estrogen-hypersensitive (long-term estrogen-deprived) MCF-7 cells (190;191). Indeed, Chan et al. have previously measured SMRT mRNA levels in a cohort of 19 tamoxifen-resistant tumors, and they showed no significant differences compared with tamoxifen-treated or untreated tumors (not selected for resistance) (190). Peterson et al showed that depletion of SMRT did not increase the agonist potential of 4HT on ER activity in MCF-7 breast cancer cells, implying that endogenous SMRT is not a significant contributor to tamoxifen's antagonist activity in this cell environment. However, they also showed that knockdown of SMRT expression in HeLa cells, did enhance ER agonist activity of 4HT, indicating that endogenous SMRT contributes to the antagonistic biocharacter of 4HT in this cell type (157). Therefore, the ability of endogenous SMRT to modulate tamoxifen activity may be "context-specific". A number of other co-repressors are potential repressors of tamoxifen-bound ER activity, including N-CoR and REA (25), and it is possible that one or more of these molecules plays a role, together with SMRT, in determining tamoxifen resistance. Furthermore, tamoxifen resistance is thought to derive from an imbalance between co-activators and co-repressors actions. Therefore additional studies investigating the co-expression of SMRT with other co-repressors and with co-activators in tamoxifen treated patients are necessary. Finally, the biological role of SMRT in ER-mediated tumorigenesis and in tamoxifen response may be more complex. Indeed, in addition to SMRT role as ER co-repressor, two recent studies reported that SMRT is also required for maximal expression of ER target genes, positively contributes to proliferation of ER-positive breast cancer cells (157) and it is recruited, in an E2-dependent manner, to the regulatory regions of PR and cyclin D1 genes (192). SMRT can also directly bind to AIB1 and plays a role in modulating the transcriptional activity of this co-activator as well as its interaction with ER target genes (192).

Here we analyzed the prognostic impact of co-expression of multiple co-activators. We observed that overexpression of any one SRC family member was associated with improved recurrence and distant recurrence free survival relative to tumors without overexpression of SRCs, whilst overexpression of two or more SRCs was associated with reduced recurrence free and distant recurrence free survival. This "concentration" dependent "yin-yang" role of the SRCs might

reflect a necessary role for SRC in normal cellular homeostasis, which becomes overridden by the SRCs role in proliferation and metastasis upon their overexpression. In any case, this exploratory analysis suggests a greater degree of interaction between SRC family members and each other, and possibly other signaling pathways, than is revealed by simple modeling of SRC expression using single markers.

Illustrative of the potentially complex interaction of SRC expression and other signaling pathways is the existence of a sub-group of breast cancers with HER2/AIB1 overexpression, which exhibit extremely poor outcome in both ER positive and ER negative breast cancers. Patients whose tumors express high levels of both HER2 and AIB1 appear to be at significantly greater risk of early relapse that those whose tumors express high levels of only one of these genes. This confirms earlier reports by Kirkegaard et al. and Osborne et al. (152;153) in ER positive tamoxifen-treated cancers, but for the first time in this study we have sufficient power to confirm the effect in a multivariate regression analysis. Although HER2/AIB1 overexpressing tumors represents only a small proportion of the total breast cancer population, this sub-group makes up 25% of HER2 positive disease and the extremely poor prognosis warrants further investigation. Moreover in the current study we observed a similar effect for ER positive HER2/SRC2 overexpressing tumors, although were unable to detect a significant effect for HER2/SRC1 overexpressing tumors (data not shown). Signaling through the HER2 receptor activates MAPK, which in turn phosphorylates not only ER but also AIB1 (193). Phosphorylation of co-activators, similarly to that of the receptor, enhances the activity of the co-activators themselves on the genomic ER, even in the absence of its ligand or in the presence of anti-estrogens (140). This phosphorylation potentiates the ability of estrogen and SERMs to interact with ER and to recruit other transcriptional co-regulators to its transcriptional complex (193). Furthermore, it can directly activate their intrinsic enzymatic activities (194). Increased agonist activity of tamoxifen- bound ER induced by co-activators might thereby reduce the clinical benefit of adjuvant tamoxifen therapy. Clearly this may explain the effects observed in ER positive disease, however, we found similar observations in ER negative breast cancers. This is more complicated to explain, but alternative signaling pathways (e.g. via AIB/AP1 mediated transcription) might act as the key driver behind this clinical observation. Further investigation of the possible signaling pathways, and the impact of treatment with Herceptin and or aromatase inhibitors in this patient population would be of value.

CONCLUSIONS

Because the tumor specimens were not derived from patients randomly assigned to either a no-adjuvant-treatment group or to an adjuvant tamoxifen group and because many of the analyses are exploratory, the results presented here are not definitive with regard to their clinical implications. However the considerable sample size gives strength to our data.

Our results suggest that:

(i) The SRC family of ER co-activators is associated with poor outcome in both ER positive and ER negative breast cancers;

(ii) Patients with tumors overexpressing both HER2 and AIB1 relapse and die significantly earlier than patients with overexpression of either marker or those with no overexpression of AIB1/HER2;

(iii) In ER negative disease both SRC1 and AIB1 are associated with reduced RFS, DRFS and OS, showing for the first time a significant impact of these ER co-regulators in non-ER expressing tumors;

(iv) Nuclear SMRT is associated with poor recurrence free survival in both ER positive and ER negative untreated breast cancers;

(v) SMRT levels are not associated with outcome in ER positive tamoxifen treated tumors.

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For SMRT study Steffi Oesterreich, Susan Hilsenbeck and Carolyn Smith form Baylor College of Medicine conceived the experimental design and were involved in the data analysis. Mao Sufeng and Jian Huang were involved in the assay development and staining of SMRT. Wu Meng-Fen performed the statistical analysis and was involved in the generation of figures.

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REFERENCES

1. Jemal, A., Siegel, R., Xu, J., and Ward, E. Cancer Statistics, 2010. CA Cancer J.Clin. 2010;60(5):277-300.

2. Jemal, A., Center, M. M., DeSantis, C., and Ward, E. M. Global Patterns of Cancer Incidence and Mortality Rates and Trends. Cancer Epidemiol.Biomarkers Prev. 2010;19(8):1893-907.

3. Jemal, A., Siegel, R., Ward, E., Hao, Y., Xu, J., Murray, T., and Thun, M. J. Cancer Statistics, 2008. CA Cancer J.Clin. 2008;58(2):71-96.

4. Jemal, A., Siegel, R., Ward, E., Murray, T., Xu, J., and Thun, M. J. Cancer Statistics, 2007. CA Cancer J.Clin. 2007;57(1):43-66.

5. Beatson, G. T. On the Etiology of Cancer, With a Note of Some Experiments. Br.Med.J 18-2-1899;1(1990):399-400.

6. Enmark, E. and Gustafsson, J. A. Oestrogen Receptors - an Overview. J.Intern.Med. 1999;246(2):133-8.

7. Pearce, S. T. and Jordan, V. C. The Biological Role of Estrogen Receptors Alpha and Beta in Cancer. Crit Rev.Oncol.Hematol. 2004;50(1):3-22.

8. Greene, G. L., Gilna, P., Waterfield, M., Baker, A., Hort, Y., and Shine, J. Sequence and Expression of Human Estrogen Receptor Complementary DNA. Science 7-3-1986;231(4742):1150-4.

9. Enmark, E., Pelto-Huikko, M., Grandien, K., Lagercrantz, S., Lagercrantz, J., Fried, G., Nordenskjold, M., and Gustafsson, J. A. Human Estrogen Receptor Beta-Gene Structure, Chromosomal Localization, and Expression Pattern. J.Clin.Endocrinol.Metab 1997;82(12):4258-65.

10. Evans, R. M. The Steroid and Thyroid Hormone Receptor Superfamily. Science 13-5-1988;240(4854):889-95.

11. Ellmann, S., Sticht, H., Thiel, F., Beckmann, M. W., Strick, R., and Strissel, P. L. Estrogen and Progesterone Receptors: From Molecular Structures to Clinical Targets. Cell Mol.Life Sci. 2009;66(15):2405-26.

12. Shiau, A. K., Barstad, D., Loria, P. M., Cheng, L., Kushner, P. J., Agard, D. A., and Greene, G. L. The Structural Basis of Estrogen Receptor/Coactivator Recognition and the Antagonism of This Interaction by Tamoxifen. Cell 23-12-1998;95(7):927-37.

13. Lin, B. C., Hong, S. H., Krig, S., Yoh, S. M., and Privalsky, M. L. A Conformational Switch in Nuclear Hormone Receptors Is Involved in Coupling Hormone Binding to Corepressor Release. Mol.Cell Biol. 1997;17(10):6131-8.

14. Aranda, A. and Pascual, A. Nuclear Hormone Receptors and Gene Expression. Physiol Rev. 2001;81(3):1269-304.

15. Chambraud, B., Berry, M., Redeuilh, G., Chambon, P., and Baulieu, E. E. Several Regions of Human Estrogen Receptor Are Involved in the Formation of Receptor-Heat Shock Protein 90 Complexes. J.Biol.Chem. 25-11-1990;265(33):20686-91.

16. Lewis, J. S. and Jordan, V. C. Selective Estrogen Receptor Modulators (SERMs): Mechanisms of Anticarcinogenesis and Drug Resistance. Mutat.Res. 11-12-2005;591(1-2):247-63.

17. O'Malley, B. W. Coregulators: From Whence Came These "Master Genes". Mol.Endocrinol. 2007;21(5):1009-13.

18. O'Malley, B. W. and Kumar, R. Nuclear Receptor Coregulators in Cancer Biology. Cancer Res. 1-11-2009;69(21):8217-22.

19. McKenna, N. J. and O'Malley, B. W. Combinatorial Control of Gene Expression by Nuclear Receptors and Coregulators. Cell 22-2-2002;108(4):465-74.

20. Glass, C. K., Rose, D. W., and Rosenfeld, M. G. Nuclear Receptor Coactivators. Curr.Opin.Cell Biol. 1997;9(2):222-32.

21. Smith, C. L. and O'Malley, B. W. Coregulator Function: a Key to Understanding Tissue Specificity of Selective Receptor Modulators. Endocr.Rev. 2004;25(1):45-71.

22. Lonard, D. M., Nawaz, Z., Smith, C. L., and O'Malley, B. W. The 26S Proteasome Is Required for Estrogen Receptor-Alpha and Coactivator Turnover and for Efficient Estrogen Receptor-Alpha Transactivation. Mol.Cell 2000;5(6):939-48.

23. Lonard, D. M. and O'Malley, B. W. Nuclear Receptor Coregulators: Judges, Juries, and Executioners of Cellular Regulation. Mol.Cell 7-9-2007;27(5):691-700.

24. Muscat, G. E., Burke, L. J., and Downes, M. The Corepressor N-CoR and Its Variants RIP13a and RIP13Delta1 Directly Interact With the Basal Transcription Factors TFIIB, TAFII32 and TAFII70. Nucleic Acids Res. 15-6-1998;26(12):2899-907.

25. Dobrzycka, K. M., Townson, S. M., Jiang, S., and Oesterreich, S. Estrogen Receptor Corepressors -- a Role in Human Breast Cancer? Endocr.Relat Cancer 2003;10(4):517-36.

26. Jepsen, K. and Rosenfeld, M. G. Biological Roles and Mechanistic Actions of Co-Repressor Complexes. J.Cell Sci. 15-2-2002;115(Pt 4):689-98.

27. Girault, I., Bieche, I., and Lidereau, R. Role of Estrogen Receptor Alpha Transcriptional Coregulators in Tamoxifen Resistance in Breast Cancer. Maturitas 20-7-2006;54(4):342-51.

28. Xu, J. and Li, Q. Review of the in Vivo Functions of the P160 Steroid Receptor Coactivator Family. Mol.Endocrinol. 2003;17(9):1681-92.

29. Leo, C. and Chen, J. D. The SRC Family of Nuclear Receptor Coactivators. Gene 7-3-2000;245(1):1-11.

30. Xu, J., Wu, R. C., and O'Malley, B. W. Normal and Cancer-Related Functions of the P160 Steroid Receptor Co-Activator (SRC) Family. Nat.Rev.Cancer 2009;9(9):615-30.

31. Chen, Y. H., Kim, J. H., and Stallcup, M. R. GAC63, a GRIP1-Dependent Nuclear Receptor Coactivator. Mol.Cell Biol. 2005;25(14):5965-72.

32. Kim, J. H., Li, H., and Stallcup, M. R. CoCoA, a Nuclear Receptor Coactivator Which Acts Through an N-Terminal Activation Domain of P160 Coactivators. Mol.Cell 2003;12(6):1537-49.

33. Lee, Y. H., Campbell, H. D., and Stallcup, M. R. Developmentally Essential Protein Flightless I Is a Nuclear Receptor Coactivator With Actin Binding Activity. Mol.Cell Biol. 2004;24(5):2103-17.

34. Heery, D. M., Kalkhoven, E., Hoare, S., and Parker, M. G. A Signature Motif in Transcriptional Co-Activators Mediates Binding to Nuclear Receptors. Nature 12-6-1997;387(6634):733-6.

35. Torchia, J., Rose, D. W., Inostroza, J., Kamei, Y., Westin, S., Glass, C. K., and Rosenfeld, M. G. The Transcriptional Co-Activator P/CIP Binds CBP and Mediates Nuclear-Receptor Function. Nature 12-6-1997;387(6634):677-84.

36. Ding, X. F., Anderson, C. M., Ma, H., Hong, H., Uht, R. M., Kushner, P. J., and Stallcup, M. R. Nuclear Receptor-Binding Sites of Coactivators Glucocorticoid Receptor Interacting Protein 1 (GRIP1) and Steroid Receptor Coactivator 1 (SRC-1): Multiple Motifs With Different Binding Specificities. Mol.Endocrinol. 1998;12(2):302-13.

37. Voegel, J. J., Heine, M. J., Zechel, C., Chambon, P., and Gronemeyer, H. TIF2, a 160 KDa Transcriptional Mediator for the Ligand-Dependent Activation Function AF-2 of Nuclear Receptors. EMBO J. 15-7-1996;15(14):3667-75.

38. Li, H. and Chen, J. D. The Receptor-Associated Coactivator 3 Activates Transcription Through CREB-Binding Protein Recruitment and Autoregulation. J.Biol.Chem. 6-3-1998;273(10):5948-54.

39. Onate, S. A., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. Sequence and Characterization of a Coactivator for the Steroid Hormone Receptor Superfamily. Science 24-11-1995;270(5240):1354-7.

40. Chen, D., Ma, H., Hong, H., Koh, S. S., Huang, S. M., Schurter, B. T., Aswad, D. W., and Stallcup, M. R. Regulation of Transcription by a Protein Methyltransferase. Science 25-6-1999;284(5423):2174-7.

41. Koh, S. S., Chen, D., Lee, Y. H., and Stallcup, M. R. Synergistic Enhancement of Nuclear Receptor Function by P160 Coactivators and Two Coactivators With Protein Methyltransferase Activities. J.Biol.Chem. 12-1-2001;276(2):1089-98.

42. Feng, Q., Yi, P., Wong, J., and O'Malley, B. W. Signaling Within a Coactivator Complex: Methylation of SRC-3/AIB1 Is a Molecular Switch for Complex Disassembly. Mol.Cell Biol. 2006;26(21):7846-57.

43. Wu, R. C., Feng, Q., Lonard, D. M., and O'Malley, B. W. SRC-3 Coactivator Functional Lifetime Is Regulated by a Phospho-Dependent Ubiquitin Time Clock. Cell 15-6-2007;129(6):1125-40.

44. Carapeti, M., Aguiar, R. C., Chase, A., Goldman, J. M., and Cross, N. C. Assignment of the Steroid Receptor Coactivator-1 (SRC-1) Gene to Human Chromosome Band 2p23. Genomics 1-9-1998;52(2):242-4.

45. Webb, P., Nguyen, P., Shinsako, J., Anderson, C., Feng, W., Nguyen, M. P., Chen, D., Huang, S. M., Subramanian, S., McKinerney, E., Katzenellenbogen, B. S., Stallcup, M. R., and Kushner, P. J. Estrogen Receptor Activation Function 1 Works by Binding P160 Coactivator Proteins. Mol.Endocrinol. 1998;12(10):1605-18.

46. Alen, P., Claessens, F., Verhoeven, G., Rombauts, W., and Peeters, B. The Androgen Receptor Amino-Terminal Domain Plays a Key Role in P160 Coactivator-Stimulated Gene Transcription. Mol.Cell Biol. 1999;19(9):6085-97.

47. Ma, H., Hong, H., Huang, S. M., Irvine, R. A., Webb, P., Kushner, P. J., Coetzee, G. A., and Stallcup, M. R. Multiple Signal Input and Output Domains of the 160-Kilodalton Nuclear Receptor Coactivator Proteins. Mol.Cell Biol. 1999;19(9):6164-73.

48. Lee, S. K., Kim, H. J., Na, S. Y., Kim, T. S., Choi, H. S., Im, S. Y., and Lee, J. W. Steroid Receptor Coactivator-1 Coactivates Activating Protein-1-Mediated Transactivations Through Interaction With the C-Jun and C-Fos Subunits. J.Biol.Chem. 3-7-1998;273(27):16651-4.

49. Yanagisawa, J., Yanagi, Y., Masuhiro, Y., Suzawa, M., Watanabe, M., Kashiwagi, K., Toriyabe, T., Kawabata, M., Miyazono, K., and Kato, S. Convergence of Transforming Growth Factor-Beta and Vitamin D Signaling Pathways on SMAD Transcriptional Coactivators. Science 26-2-1999;283(5406):1317-21.

50. Na, S. Y., Lee, S. K., Han, S. J., Choi, H. S., Im, S. Y., and Lee, J. W. Steroid Receptor Coactivator-1 Interacts With the P50 Subunit and Coactivates Nuclear Factor KappaB-Mediated Transactivations. J.Biol.Chem. 1-5-1998;273(18):10831-4.

51. Nishihara, E., Yoshida-Komiya, H., Chan, C. S., Liao, L., Davis, R. L., O'Malley, B. W., and Xu, J. SRC-1 Null Mice Exhibit Moderate Motor Dysfunction and Delayed Development of Cerebellar Purkinje Cells. J.Neurosci. 1-1-2003;23(1):213-22.

52. Xu, J., Qiu, Y., DeMayo, F. J., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. Partial Hormone Resistance in Mice With Disruption of the Steroid Receptor Coactivator-1 (SRC-1) Gene. Science 20-3-1998;279(5358):1922-5.

53. Fleming, F. J., Hill, A. D., McDermott, E. W., O'Higgins, N. J., and Young, L. S. Differential Recruitment of Coregulator Proteins Steroid Receptor Coactivator-1 and Silencing Mediator for Retinoid and Thyroid Receptors to the Estrogen Receptor-Estrogen Response Element by Beta-Estradiol and 4-Hydroxytamoxifen in Human Breast Cancer. J.Clin.Endocrinol.Metab 2004;89(1):375-83.

54. Tai, H., Kubota, N., and Kato, S. Involvement of Nuclear Receptor Coactivator SRC-1 in Estrogen-Dependent Cell Growth of MCF-7 Cells. Biochem.Biophys.Res.Commun. 7-1-2000;267(1):311-6.

55. Cavarretta, I. T., Mukopadhyay, R., Lonard, D. M., Cowsert, L. M., Bennett, C. F., O'Malley, B. W., and Smith, C. L. Reduction of Coactivator Expression by Antisense Oligodeoxynucleotides Inhibits ERalpha Transcriptional Activity and MCF-7 Proliferation. Mol.Endocrinol. 2002;16(2):253-70.

56. Kishimoto, H., Wang, Z., Bhat-Nakshatri, P., Chang, D., Clarke, R., and Nakshatri, H. The P160 Family Coactivators Regulate Breast Cancer Cell Proliferation and Invasion Through Autocrine/Paracrine Activity of SDF-1alpha/CXCL12. Carcinogenesis 2005;26(10):1706-15.

57. Wang, S., Yuan, Y., Liao, L., Kuang, S. Q., Tien, J. C., O'Malley, B. W., and Xu, J. Disruption of the SRC-1 Gene in Mice Suppresses Breast Cancer Metastasis Without Affecting Primary Tumor Formation. Proc.Natl.Acad.Sci.U.S.A 6-1-2009;106(1):151-6.

58. Qin, L., Liu, Z., Chen, H., and Xu, J. The Steroid Receptor Coactivator-1 Regulates Twist Expression and Promotes Breast Cancer Metastasis. Cancer Res. 1-5-2009;69(9):3819-27.

59. Ning, G., Jurecic, V., Baldini, A., and Xu, J. Structure and Chromosomal Locations of Mouse Steroid Receptor Coactivator Gene Family. In Vitro Cell Dev.Biol.Anim 1999;35(8):481-6.

60. Gehin, M., Mark, M., Dennefeld, C., Dierich, A., Gronemeyer, H., and Chambon, P. The Function of TIF2/GRIP1 in Mouse Reproduction Is Distinct From Those of SRC-1 and P/CIP. Mol.Cell Biol. 2002;22(16):5923-37.

61. Guan, X. Y., Xu, J., Anzick, S. L., Zhang, H., Trent, J. M., and Meltzer, P. S. Hybrid Selection of Transcribed Sequences From Microdissected DNA: Isolation of Genes Within Amplified Region at 20q11-Q13.2 in Breast Cancer. Cancer Res. 1-8-1996;56(15):3446-50.

62. Anzick, S. L., Kononen, J., Walker, R. L., Azorsa, D. O., Tanner, M. M., Guan, X. Y., Sauter, G., Kallioniemi, O. P., Trent, J. M., and Meltzer, P. S. AIB1, a Steroid Receptor Coactivator Amplified in Breast and Ovarian Cancer. Science 15-8-1997;277(5328):965-8.

63. Goel, A. and Janknecht, R. Concerted Activation of ETS Protein ER81 by P160 Coactivators, the Acetyltransferase P300 and the Receptor Tyrosine Kinase HER2/Neu. J.Biol.Chem. 9-4-2004;279(15):14909-16.

64. Mussi, P., Yu, C., O'Malley, B. W., and Xu, J. Stimulation of Steroid Receptor Coactivator-3 (SRC-3) Gene Overexpression by a Positive Regulatory Loop of E2F1 and SRC-3. Mol.Endocrinol. 2006;20(12):3105-19.

65. Xu, J., Liao, L., Ning, G., Yoshida-Komiya, H., Deng, C., and O'Malley, B. W. The Steroid Receptor Coactivator SRC-3 (p/CIP/RAC3/AIB1/ACTR/TRAM-1) Is Required for Normal Growth, Puberty, Female Reproductive Function, and Mammary Gland Development. Proc.Natl.Acad.Sci.U.S.A 6-6-2000;97(12):6379-84.

66. Louie, M. C., Zou, J. X., Rabinovich, A., and Chen, H. W. ACTR/AIB1 Functions As an E2F1 Coactivator to Promote Breast Cancer Cell Proliferation and Antiestrogen Resistance. Mol.Cell Biol. 2004;24(12):5157-71.

67. Oh, A., List, H. J., Reiter, R., Mani, A., Zhang, Y., Gehan, E., Wellstein, A., and Riegel, A. T. The Nuclear Receptor Coactivator AIB1 Mediates Insulin-Like Growth Factor I-Induced Phenotypic Changes in Human Breast Cancer Cells. Cancer Res. 15-11-2004;64(22):8299-308.

68. Planas-Silva, M. D., Shang, Y., Donaher, J. L., Brown, M., and Weinberg, R. A. AIB1 Enhances Estrogen-Dependent Induction of Cyclin D1 Expression. Cancer Res. 15-5-2001;61(10):3858-62.

69. Lahusen, T., Fereshteh, M., Oh, A., Wellstein, A., and Riegel, A. T. Epidermal Growth Factor Receptor Tyrosine Phosphorylation and Signaling Controlled by a Nuclear Receptor Coactivator, Amplified in Breast Cancer 1. Cancer Res. 1-8-2007;67(15):7256-65.

70. List, H. J., Lauritsen, K. J., Reiter, R., Powers, C., Wellstein, A., and Riegel, A. T. Ribozyme Targeting Demonstrates That the Nuclear Receptor Coactivator AIB1 Is a Rate-Limiting Factor for Estrogen-Dependent Growth of Human MCF-7 Breast Cancer Cells. J.Biol.Chem. 29-6-2001;276(26):23763-8.

71. Kuang, S. Q., Liao, L., Zhang, H., Lee, A. V., O'Malley, B. W., and Xu, J. AIB1/SRC-3 Deficiency Affects Insulin-Like Growth Factor I Signaling Pathway and Suppresses V-Ha-Ras-Induced Breast Cancer Initiation and Progression in Mice. Cancer Res. 1-3-2004;64(5):1875-85.

72. Fereshteh, M. P., Tilli, M. T., Kim, S. E., Xu, J., O'Malley, B. W., Wellstein, A., Furth, P. A., and Riegel, A. T. The Nuclear Receptor Coactivator Amplified in Breast Cancer-1 Is Required for Neu (ErbB2/HER2) Activation, Signaling, and Mammary Tumorigenesis in Mice. Cancer Res. 15-5-2008;68(10):3697-706.

73. Kuang, S. Q., Liao, L., Wang, S., Medina, D., O'Malley, B. W., and Xu, J. Mice Lacking the Amplified in Breast Cancer 1/Steroid Receptor Coactivator-3 Are Resistant to Chemical Carcinogen-Induced Mammary Tumorigenesis. Cancer Res. 1-9-2005;65(17):7993-8002.

74. Qin, L., Liao, L., Redmond, A., Young, L., Yuan, Y., Chen, H., O'Malley, B. W., and Xu, J. The AIB1 Oncogene Promotes Breast Cancer Metastasis by Activation of PEA3-Mediated Matrix

Metalloproteinase 2 (MMP2) and MMP9 Expression. Mol.Cell Biol. 2008;28(19):5937-50.

75. Tilli, M. T., Reiter, R., Oh, A. S., Henke, R. T., McDonnell, K., Gallicano, G. I., Furth, P. A., and Riegel, A. T. Overexpression of an N-Terminally Truncated Isoform of the Nuclear Receptor Coactivator Amplified in Breast Cancer 1 Leads to Altered Proliferation of Mammary Epithelial Cells in Transgenic Mice. Mol.Endocrinol. 2005;19(3):644-56.

76. Torres-Arzayus, M. I., Font, de Mora, Yuan, J., Vazquez, F., Bronson, R., Rue, M., Sellers, W. R., and Brown, M. High Tumor Incidence and Activation of the PI3K/AKT Pathway in Transgenic Mice Define AIB1 As an Oncogene. Cancer Cell 2004;6(3):263-74.

77. Stanya, K. J. and Kao, H. Y. New Insights into the Functions and Regulation of the Transcriptional Corepressors SMRT and N-CoR. Cell Div. 2009;4:7.

78. Horlein, A. J., Naar, A. M., Heinzel, T., Torchia, J., Gloss, B., Kurokawa, R., Ryan, A., Kamei, Y., Soderstrom, M., Glass, C. K., and . Ligand-Independent Repression by the Thyroid Hormone Receptor Mediated by a Nuclear Receptor Co-Repressor. Nature 5-10-1995;377(6548):397-404.

79. Chen, J. D. and Evans, R. M. A Transcriptional Co-Repressor That Interacts With Nuclear Hormone Receptors. Nature 5-10-1995;377(6548):454-7.

80. Ordentlich, P., Downes, M., Xie, W., Genin, A., Spinner, N. B., and Evans, R. M. Unique Forms of Human and Mouse Nuclear Receptor Corepressor SMRT. Proc.Natl.Acad.Sci.U.S.A 16-3-1999;96(6):2639-44.

81. Park, E. J., Schroen, D. J., Yang, M., Li, H., Li, L., and Chen, J. D. SMRTe, a Silencing Mediator for Retinoid and Thyroid Hormone Receptors-Extended Isoform That Is More Related to the Nuclear Receptor Corepressor. Proc.Natl.Acad.Sci.U.S.A 30-3-1999;96(7):3519-24.

82. Jenster, G. Coactivators and Corepressors As Mediators of Nuclear Receptor Function: an Update. Mol.Cell Endocrinol. 25-8-1998;143(1-2):1-7.

83. Koenig, R. J. Thyroid Hormone Receptor Coactivators and Corepressors. Thyroid 1998;8(8):703-13.

84. Lee, J. W., Lee, Y. C., Na, S. Y., Jung, D. J., and Lee, S. K. Transcriptional Coregulators of the Nuclear Receptor Superfamily: Coactivators and Corepressors. Cell Mol.Life Sci. 2001;58(2):289-97.

85. Jones, P. L. and Shi, Y. B. N-CoR-HDAC Corepressor Complexes: Roles in Transcriptional Regulation by Nuclear Hormone Receptors. Curr.Top.Microbiol.Immunol. 2003;274:237-68.

86. Torchia, J., Glass, C., and Rosenfeld, M. G. Co-Activators and Co-Repressors in the Integration of Transcriptional Responses. Curr.Opin.Cell Biol. 1998;10(3):373-83.

87. Seol, W., Mahon, M. J., Lee, Y. K., and Moore, D. D. Two Receptor Interacting Domains in the Nuclear Hormone Receptor Corepressor RIP13/N-CoR. Mol.Endocrinol. 1996;10(12):1646-55.

88. Downes, M., Burke, L. J., Bailey, P. J., and Muscat, G. E. Two Receptor Interaction Domains in the Corepressor, N-CoR/RIP13, Are Required for an Efficient Interaction With Rev-ErbA Alpha and RVR: Physical Association Is Dependent on the E Region of the Orphan Receptors. Nucleic Acids Res. 15-11-1996;24(22):4379-86.

89. Zamir, I., Dawson, J., Lavinsky, R. M., Glass, C. K., Rosenfeld, M. G., and Lazar, M. A. Cloning and Characterization of a Corepressor and Potential Component of the Nuclear Hormone Receptor Repression Complex. Proc.Natl.Acad.Sci.U.S.A 23-12-1997;94(26):14400-5.

90. Webb, P., Anderson, C. M., Valentine, C., Nguyen, P., Marimuthu, A., West, B. L., Baxter, J. D., and Kushner, P. J. The Nuclear Receptor Corepressor (N-CoR) Contains Three Isoleucine Motifs (I/LXXII) That Serve As Receptor Interaction Domains (IDs). Mol.Endocrinol. 2000;14(12):1976-85.

91. Cohen, R. N., Brzostek, S., Kim, B., Chorev, M., Wondisford, F. E., and Hollenberg, A. N. The Specificity of Interactions Between Nuclear

Hormone Receptors and Corepressors Is Mediated by Distinct Amino Acid Sequences Within the Interacting Domains. Mol.Endocrinol. 2001;15(7):1049-61.

92. Hu, X. and Lazar, M. A. The CoRNR Motif Controls the Recruitment of Corepressors by Nuclear Hormone Receptors. Nature 4-11-1999;402(6757):93-6.

93. Perissi, V., Staszewski, L. M., McInerney, E. M., Kurokawa, R., Krones, A., Rose, D. W., Lambert, M. H., Milburn, M. V., Glass, C. K., and Rosenfeld, M. G. Molecular Determinants of Nuclear Receptor-Corepressor Interaction. Genes Dev. 15-12-1999;13(24):3198-208.

94. Sande, S. and Privalsky, M. L. Identification of TRACs (T3 Receptor-Associating Cofactors), a Family of Cofactors That Associate With, and Modulate the Activity of, Nuclear Hormone Receptors. Mol.Endocrinol. 1996;10(7):813-25.

95. Dotzlaw, H., Moehren, U., Mink, S., Cato, A. C., Iniguez Lluhi, J. A., and Baniahmad, A. The Amino Terminus of the Human AR Is Target for Corepressor Action and Antihormone Agonism. Mol.Endocrinol. 2002;16(4):661-73.

96. Jackson, T. A., Richer, J. K., Bain, D. L., Takimoto, G. S., Tung, L., and Horwitz, K. B. The Partial Agonist Activity of Antagonist-Occupied Steroid Receptors Is Controlled by a Novel Hinge Domain-Binding Coactivator L7/SPA and the Corepressors N-CoR or SMRT. Mol.Endocrinol. 1997;11(6):693-705.

97. Smith, C. L., Nawaz, Z., and O'Malley, B. W. Coactivator and Corepressor Regulation of the Agonist/Antagonist Activity of the Mixed Antiestrogen, 4-Hydroxytamoxifen. Mol.Endocrinol. 1997;11(6):657-66.

98. Takimoto, G. S., Graham, J. D., Jackson, T. A., Tung, L., Powell, R. L., Horwitz, L. D., and Horwitz, K. B. Tamoxifen Resistant Breast Cancer: Coregulators Determine the Direction of Transcription by Antagonist-Occupied Steroid Receptors. J.Steroid Biochem.Mol.Biol. 1999;69(1-6):45-50.

99. Zhang, X., Jeyakumar, M., Petukhov, S., and Bagchi, M. K. A Nuclear Receptor Corepressor Modulates Transcriptional Activity of

Antagonist-Occupied Steroid Hormone Receptor. Mol.Endocrinol. 1998;12(4):513-24.

100. Brzozowski, A. M., Pike, A. C., Dauter, Z., Hubbard, R. E., Bonn, T., Engstrom, O., Ohman, L., Greene, G. L., Gustafsson, J. A., and Carlquist, M. Molecular Basis of Agonism and Antagonism in the Oestrogen Receptor. Nature 16-10-1997;389(6652):753-8.

101. Paige, L. A., Christensen, D. J., Gron, H., Norris, J. D., Gottlin, E. B., Padilla, K. M., Chang, C. Y., Ballas, L. M., Hamilton, P. T., McDonnell, D. P., and Fowlkes, D. M. Estrogen Receptor (ER) Modulators Each Induce Distinct Conformational Changes in ER Alpha and ER Beta. Proc.Natl.Acad.Sci.U.S.A 30-3-1999;96(7):3999-4004.

102. Wong, C. W. and Privalsky, M. L. Transcriptional Repression by the SMRT-MSin3 Corepressor: Multiple Interactions, Multiple Mechanisms, and a Potential Role for TFIIB. Mol.Cell Biol. 1998;18(9):5500-10.

103. Zhou, Y., Gross, W., Hong, S. H., and Privalsky, M. L. The SMRT Corepressor Is a Target of Phosphorylation by Protein Kinase CK2 (Casein Kinase II). Mol.Cell Biochem. 2001;220(1-2):1-13.

104. Hong, S. H., Wong, C. W., and Privalsky, M. L. Signaling by Tyrosine Kinases Negatively Regulates the Interaction Between Transcription Factors and SMRT (Silencing Mediator of Retinoic Acid and Thyroid Hormone Receptor) Corepressor. Mol.Endocrinol. 1998;12(8):1161-71.

105. Hong, S. H. and Privalsky, M. L. The SMRT Corepressor Is Regulated by a MEK-1 Kinase Pathway: Inhibition of Corepressor Function Is Associated With SMRT Phosphorylation and Nuclear Export. Mol.Cell Biol. 2000;20(17):6612-25.

106. Jepsen, K., Solum, D., Zhou, T., McEvilly, R. J., Kim, H. J., Glass, C. K., Hermanson, O., and Rosenfeld, M. G. SMRT-Mediated Repression of an H3K27 Demethylase in Progression From Neural Stem Cell to Neuron. Nature 15-11-2007;450(7168):415-9.

107. Jepsen, K., Gleiberman, A. S., Shi, C., Simon, D. I., and Rosenfeld, M. G. Cooperative Regulation in Development by SMRT and FOXP1. Genes Dev. 15-3-2008;22(6):740-5.

108. Jepsen, K., Hermanson, O., Onami, T. M., Gleiberman, A. S., Lunyak, V., McEvilly, R. J., Kurokawa, R., Kumar, V., Liu, F., Seto, E., Hedrick, S. M., Mandel, G., Glass, C. K., Rose, D. W., and Rosenfeld, M. G. Combinatorial Roles of the Nuclear Receptor Corepressor in Transcription and Development. Cell 15-9-2000;102(6):753-63.

109. Keeton, E. K. and Brown, M. Cell Cycle Progression Stimulated by Tamoxifen-Bound Estrogen Receptor-Alpha and Promoter-Specific Effects in Breast Cancer Cells Deficient in N-CoR and SMRT. Mol.Endocrinol. 2005;19(6):1543-54.

110. Stanya, K. J., Liu, Y., Means, A. R., and Kao, H. Y. Cdk2 and Pin1 Negatively Regulate the Transcriptional Corepressor SMRT. J.Cell Biol. 6-10-2008;183(1):49-61.

111. Fleming, F. J., Myers, E., Kelly, G., Crotty, T. B., McDermott, E. W., O'Higgins, N. J., Hill, A. D., and Young, L. S. Expression of SRC-1, AIB1, and PEA3 in HER2 Mediated Endocrine Resistant Breast Cancer; a Predictive Role for SRC-1. J.Clin.Pathol. 2004;57(10):1069-74.

112. Myers, E., Hill, A. D., Kelly, G., McDermott, E. W., O'Higgins, N. J., Buggy, Y., and Young, L. S. Associations and Interactions Between Ets-1 and Ets-2 and Coregulatory Proteins, SRC-1, AIB1, and NCoR in Breast Cancer. Clin.Cancer Res. 15-3-2005;11(6):2111-22.

113. Hudelist, G., Czerwenka, K., Kubista, E., Marton, E., Pischinger, K., and Singer, C. F. Expression of Sex Steroid Receptors and Their Co-Factors in Normal and Malignant Breast Tissue: AIB1 Is a Carcinoma-Specific Co-Activator. Breast Cancer Res.Treat. 2003;78(2):193-204.

114. Redmond, A. M., Bane, F. T., Stafford, A. T., McIlroy, M., Dillon, M. F., Crotty, T. B., Hill, A. D., and Young, L. S. Coassociation of Estrogen Receptor and P160 Proteins Predicts Resistance to

Endocrine Treatment; SRC-1 Is an Independent Predictor of Breast Cancer Recurrence. Clin.Cancer Res. 15-3-2009;15(6):2098-106.

115. List, H. J., Reiter, R., Singh, B., Wellstein, A., and Riegel, A. T. Expression of the Nuclear Coactivator AIB1 in Normal and Malignant Breast Tissue. Breast Cancer Res.Treat. 2001;68(1):21-8.

116. Green, A. R., Burney, C., Granger, C. J., Paish, E. C., El Sheikh, S., Rakha, E. A., Powe, D. G., Macmillan, R. D., Ellis, I. O., and Stylianou, E. The Prognostic Significance of Steroid Receptor Co-Regulators in Breast Cancer: Co-Repressor NCOR2/SMRT Is an Independent Indicator of Poor Outcome. Breast Cancer Res.Treat. 2008;110(3):427-37.

117. Myers, E., Fleming, F. J., Crotty, T. B., Kelly, G., McDermott, E. W., O'Higgins, N. J., Hill, A. D., and Young, L. S. Inverse Relationship Between ER-Beta and SRC-1 Predicts Outcome in Endocrine-Resistant Breast Cancer. Br.J.Cancer 1-11-2004;91(9):1687-93.

118. Zhao, C., Yasui, K., Lee, C. J., Kurioka, H., Hosokawa, Y., Oka, T., and Inazawa, J. Elevated Expression Levels of NCOA3, TOP1, and TFAP2C in Breast Tumors As Predictors of Poor Prognosis. Cancer 1-7-2003;98(1):18-23.

119. Bouras, T., Southey, M. C., and Venter, D. J. Overexpression of the Steroid Receptor Coactivator AIB1 in Breast Cancer Correlates With the Absence of Estrogen and Progesterone Receptors and Positivity for P53 and HER2/Neu. Cancer Res. 1-2-2001;61(3):903-7.

120. Iwase, H., Omoto, Y., Toyama, T., Yamashita, H., Hara, Y., Sugiura, H., and Zhang, Z. Clinical Significance of AIB1 Expression in Human Breast Cancer. Breast Cancer Res.Treat. 2003;80(3):339-45.

121. Bautista, S., Valles, H., Walker, R. L., Anzick, S., Zeillinger, R., Meltzer, P., and Theillet, C. In Breast Cancer, Amplification of the Steroid Receptor Coactivator Gene AIB1 Is Correlated With Estrogen and Progesterone Receptor Positivity. Clin.Cancer Res. 1998;4(12):2925-9.

122. Girault, I., Lerebours, F., Amarir, S., Tozlu, S., Tubiana-Hulin, M., Lidereau, R., and Bieche, I. Expression Analysis of Estrogen Receptor Alpha Coregulators in Breast Carcinoma: Evidence That NCOR1 Expression Is Predictive of the Response to Tamoxifen. Clin.Cancer Res. 2003;9(4):1259-66.

123. Osborne, C. K. Tamoxifen in the Treatment of Breast Cancer. N.Engl.J.Med. 26-11-1998;339(22):1609-18.

124. Jordan, V. C. Chemoprevention of Breast Cancer With Selective Oestrogen-Receptor Modulators. Nat.Rev.Cancer 2007;7(1):46-53.

125. Osborne, C. K., Coronado, E., Allred, D. C., Wiebe, V., and DeGregorio, M. Acquired Tamoxifen Resistance: Correlation With Reduced Breast Tumor Levels of Tamoxifen and Isomerization of Trans-4-Hydroxytamoxifen. J.Natl.Cancer Inst. 16-10-1991;83(20):1477-82.

126. Osborne, C. K., Wiebe, V. J., McGuire, W. L., Ciocca, D. R., and DeGregorio, M. W. Tamoxifen and the Isomers of 4-Hydroxytamoxifen in Tamoxifen-Resistant Tumors From Breast Cancer Patients. J.Clin.Oncol. 1992;10(2):304-10.

127. Wiebe, V. J., Osborne, C. K., McGuire, W. L., and DeGregorio, M. W. Identification of Estrogenic Tamoxifen Metabolite(s) in Tamoxifen-Resistant Human Breast Tumors. J.Clin.Oncol. 1992;10(6):990-4.

128. Howell, A., DeFriend, D., Robertson, J., Blamey, R., and Walton, P. Response to a Specific Antioestrogen (ICI 182780) in Tamoxifen-Resistant Breast Cancer. Lancet 7-1-1995;345(8941):29-30.

129. Sainsbury, J. R., Farndon, J. R., Sherbet, G. V., and Harris, A. L. Epidermal-Growth-Factor Receptors and Oestrogen Receptors in Human Breast Cancer. Lancet 16-2-1985;1(8425):364-6.

130. Koga, M., Musgrove, E. A., and Sutherland, R. L. Modulation of the Growth-Inhibitory Effects of Progestins and the Antiestrogen Hydroxyclomiphene on Human Breast Cancer Cells by Epidermal Growth Factor and Insulin. Cancer Res. 1-1-1989;49(1):112-6.

131. Arpino, G., Green, S. J., Allred, D. C., Lew, D., Martino, S., Osborne, C. K., and Elledge, R. M. HER-2 Amplification, HER-1 Expression,

and Tamoxifen Response in Estrogen Receptor-Positive Metastatic Breast Cancer: a Southwest Oncology Group Study. Clin.Cancer Res. 1-9-2004;10(17):5670-6.

132. De Laurentiis, M., Arpino, G., Massarelli, E., Ruggiero, A., Carlomagno, C., Ciardiello, F., Tortora, G., D'Agostino, D., Caputo, F., Cancello, G., Montagna, E., Malorni, L., Zinno, L., Lauria, R., Bianco, A. R., and De Placido, S. A Meta-Analysis on the Interaction Between HER-2 Expression and Response to Endocrine Treatment in Advanced Breast Cancer. Clin.Cancer Res. 1-7-2005;11(13):4741-8.

133. McClelland, R. A., Barrow, D., Madden, T. A., Dutkowski, C. M., Pamment, J., Knowlden, J. M., Gee, J. M., and Nicholson, R. I. Enhanced Epidermal Growth Factor Receptor Signaling in MCF7 Breast Cancer Cells After Long-Term Culture in the Presence of the Pure Antiestrogen ICI 182,780 (Faslodex). Endocrinology 2001;142(7):2776-88.

134. Knowlden, J. M., Hutcheson, I. R., Jones, H. E., Madden, T., Gee, J. M., Harper, M. E., Barrow, D., Wakeling, A. E., and Nicholson, R. I. Elevated Levels of Epidermal Growth Factor Receptor/c-ErbB2 Heterodimers Mediate an Autocrine Growth Regulatory Pathway in Tamoxifen-Resistant MCF-7 Cells. Endocrinology 2003;144(3):1032-44.

135. Hutcheson, I. R., Knowlden, J. M., Madden, T. A., Barrow, D., Gee, J. M., Wakeling, A. E., and Nicholson, R. I. Oestrogen Receptor-Mediated Modulation of the EGFR/MAPK Pathway in Tamoxifen-Resistant MCF-7 Cells. Breast Cancer Res.Treat. 2003;81(1):81-93.

136. Faridi, J., Wang, L., Endemann, G., and Roth, R. A. Expression of Constitutively Active Akt-3 in MCF-7 Breast Cancer Cells Reverses the Estrogen and Tamoxifen Responsivity of These Cells in Vivo. Clin.Cancer Res. 1-8-2003;9(8):2933-9.

137. deGraffenried, L. A., Friedrichs, W. E., Russell, D. H., Donzis, E. J., Middleton, A. K., Silva, J. M., Roth, R. A., and Hidalgo, M. Inhibition of MTOR Activity Restores Tamoxifen Response in Breast Cancer Cells With Aberrant Akt Activity. Clin.Cancer Res. 1-12-2004;10(23):8059-67.

138. Miller, T. W., Perez-Torres, M., Narasanna, A., Guix, M., Stal, O., Perez-Tenorio, G., Gonzalez-Angulo, A. M., Hennessy, B. T., Mills, G. B., Kennedy, J. P., Lindsley, C. W., and Arteaga, C. L. Loss of Phosphatase and Tensin Homologue Deleted on Chromosome 10 Engages ErbB3 and Insulin-Like Growth Factor-I Receptor Signaling to Promote Antiestrogen Resistance in Breast Cancer. Cancer Res. 15-5-2009;69(10):4192-201.

139. Arpino, G., Wiechmann, L., Osborne, C. K., and Schiff, R. Crosstalk Between the Estrogen Receptor and the HER Tyrosine Kinase Receptor Family: Molecular Mechanism and Clinical Implications for Endocrine Therapy Resistance. Endocr.Rev. 2008;29(2):217-33.

140. Schiff, R., Massarweh, S., Shou, J., and Osborne, C. K. Breast Cancer Endocrine Resistance: How Growth Factor Signaling and Estrogen Receptor Coregulators Modulate Response. Clin.Cancer Res. 2003;9(1 Pt 2):447S-54S.

141. Kato, S., Endoh, H., Masuhiro, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., Masushige, S., Gotoh, Y., Nishida, E., Kawashima, H., Metzger, D., and Chambon, P. Activation of the Estrogen Receptor Through Phosphorylation by Mitogen-Activated Protein Kinase. Science 1-12-1995;270(5241):1491-4.

142. Balasenthil, S., Barnes, C. J., Rayala, S. K., and Kumar, R. Estrogen Receptor Activation at Serine 305 Is Sufficient to Upregulate Cyclin D1 in Breast Cancer Cells. FEBS Lett. 4-6-2004;567(2-3):243-7.

143. Weigel, N. L. and Zhang, Y. Ligand-Independent Activation of Steroid Hormone Receptors. J.Mol.Med. 1998;76(7):469-79.

144. Campbell, R. A., Bhat-Nakshatri, P., Patel, N. M., Constantinidou, D., Ali, S., and Nakshatri, H. Phosphatidylinositol 3-Kinase/AKT-Mediated Activation of Estrogen Receptor Alpha: a New Model for Anti-Estrogen Resistance. J.Biol.Chem. 30-3-2001;276(13):9817-24.

145. Scott, D. J., Parkes, A. T., Ponchel, F., Cummings, M., Poola, I., and Speirs, V. Changes in Expression of Steroid Receptors, Their Downstream Target Genes and Their Associated Co-Regulators During the Sequential Acquisition of Tamoxifen Resistance in Vitro. Int.J.Oncol. 2007;31(3):557-65.

146. Shang, Y. and Brown, M. Molecular Determinants for the Tissue Specificity of SERMs. Science 29-3-2002;295(5564):2465-8.

147. Romano, A., Adriaens, M., Kuenen, S., Delvoux, B., Dunselman, G., Evelo, C., and Groothuis, P. Identification of Novel ER-Alpha Target Genes in Breast Cancer Cells: Gene- and Cell-Selective Co-Regulator Recruitment at Target Promoters Determines the Response to 17beta-Estradiol and Tamoxifen. Mol.Cell Endocrinol. 15-1-2010;314(1):90-100.

148. Berns, E. M., van Staveren, I. L., Klijn, J. G., and Foekens, J. A. Predictive Value of SRC-1 for Tamoxifen Response of Recurrent Breast Cancer. Breast Cancer Res.Treat. 1998;48(1):87-92.

149. Reiter, R., Oh, A. S., Wellstein, A., and Riegel, A. T. Impact of the Nuclear Receptor Coactivator AIB1 Isoform AIB1-Delta3 on Estrogenic Ligands With Different Intrinsic Activity. Oncogene 15-1-2004;23(2):403-9.

150. Shou, J., Massarweh, S., Osborne, C. K., Wakeling, A. E., Ali, S., Weiss, H., and Schiff, R. Mechanisms of Tamoxifen Resistance: Increased Estrogen Receptor-HER2/Neu Cross-Talk in ER/HER2-Positive Breast Cancer. J.Natl.Cancer Inst. 16-6-2004;96(12):926-35.

151. Hurtado, A., Holmes, K. A., Geistlinger, T. R., Hutcheson, I. R., Nicholson, R. I., Brown, M., Jiang, J., Howat, W. J., Ali, S., and Carroll, J. S. Regulation of ERBB2 by Oestrogen Receptor-PAX2 Determines Response to Tamoxifen. Nature 4-12-2008;456(7222):663-6.

152. Kirkegaard, T., McGlynn, L. M., Campbell, F. M., Muller, S., Tovey, S. M., Dunne, B., Nielsen, K. V., Cooke, T. G., and Bartlett, J. M. Amplified in Breast Cancer 1 in Human Epidermal Growth Factor Receptor - Positive Tumors of Tamoxifen-Treated Breast Cancer Patients. Clin.Cancer Res. 1-3-2007;13(5):1405-11.

153. Osborne, C. K., Bardou, V., Hopp, T. A., Chamness, G. C., Hilsenbeck, S. G., Fuqua, S. A., Wong, J., Allred, D. C., Clark, G. M., and Schiff, R. Role of the Estrogen Receptor Coactivator AIB1 (SRC-3) and HER-2/Neu in Tamoxifen Resistance in Breast Cancer. J.Natl.Cancer Inst. 5-3-2003;95(5):353-61.

154. Lavinsky, R. M., Jepsen, K., Heinzel, T., Torchia, J., Mullen, T. M., Schiff, R., Del Rio, A. L., Ricote, M., Ngo, S., Gemsch, J., Hilsenbeck, S. G., Osborne, C. K., Glass, C. K., Rosenfeld, M. G., and Rose, D. W. Diverse Signaling Pathways Modulate Nuclear Receptor Recruitment of N-CoR and SMRT Complexes. Proc.Natl.Acad.Sci.U.S.A 17-3-1998;95(6):2920-5.

155. Liu, X. F. and Bagchi, M. K. Recruitment of Distinct Chromatin-Modifying Complexes by Tamoxifen-Complexed Estrogen Receptor at Natural Target Gene Promoters in Vivo. J.Biol.Chem. 9-4-2004;279(15):15050-8.

156. Shang, Y., Hu, X., DiRenzo, J., Lazar, M. A., and Brown, M. Cofactor Dynamics and Sufficiency in Estrogen Receptor-Regulated Transcription. Cell 8-12-2000;103(6):843-52.

157. Peterson, T. J., Karmakar, S., Pace, M. C., Gao, T., and Smith, C. L. The Silencing Mediator of Retinoic Acid and Thyroid Hormone Receptor (SMRT) Corepressor Is Required for Full Estrogen Receptor Alpha Transcriptional Activity. Mol.Cell Biol. 2007;27(17):5933-48.

158. Graham, J. D., Bain, D. L., Richer, J. K., Jackson, T. A., Tung, L., and Horwitz, K. B. Nuclear Receptor Conformation, Coregulators, and Tamoxifen-Resistant Breast Cancer. Steroids 2000;65(10-11):579-84.

159. Graham, J. D., Bain, D. L., Richer, J. K., Jackson, T. A., Tung, L., and Horwitz, K. B. Thoughts on Tamoxifen Resistant Breast Cancer. Are Coregulators the Answer or Just a Red Herring? J.Steroid Biochem.Mol.Biol. 30-11-2000;74(5):255-9.

160. Yamamoto, Y., Wada, O., Suzawa, M., Yogiashi, Y., Yano, T., Kato, S., and Yanagisawa, J. The Tamoxifen-Responsive Estrogen Receptor Alpha Mutant D351Y Shows Reduced Tamoxifen-Dependent Interaction With Corepressor Complexes. J.Biol.Chem. 16-11-2001;276(46):42684-91.

161. Hong, W., Chen, L., Li, J., and Yao, Z. Inhibition of MAP Kinase Promotes the Recruitment of Corepressor SMRT by Tamoxifen-Bound Estrogen Receptor Alpha and Potentiates Tamoxifen Action

in MCF-7 Cells. Biochem.Biophys.Res.Commun. 28-5-2010;396(2):299-303.

162. Varlakhanova, N., Hahm, J. B., and Privalsky, M. L. Regulation of SMRT Corepressor Dimerization and Composition by MAP Kinase Phosphorylation. Mol.Cell Endocrinol. 19-10-2010.

163. Leyland-Jones, B. R., Ambrosone, C. B., Bartlett, J., Ellis, M. J., Enos, R. A., Raji, A., Pins, M. R., Zujewski, J. A., Hewitt, S. M., Forbes, J. F., Abramovitz, M., Braga, S., Cardoso, F., Harbeck, N., Denkert, C., and Jewell, S. D. Recommendations for Collection and Handling of Specimens From Group Breast Cancer Clinical Trials. J.Clin.Oncol. 1-12-2008;26(34):5638-44.

164. Allred DC, Clark GM Tandon AK McGuire W. Immunohistochemistry on Histological Sections from Small (50 mg) Samples of Pulverized Breast Cancer. J Histotechn 16[2], 117-120. 1993.
Ref Type: Generic

165. Mohsin, S. K., Weiss, H., Havighurst, T., Clark, G. M., Berardo, M., Roanh, le D., To, T. V., Qian, Z., Love, R. R., and Allred, D. C. Progesterone Receptor by Immunohistochemistry and Clinical Outcome in Breast Cancer: a Validation Study. Mod.Pathol. 2004;17(12):1545-54.

166. Harvey, J. M., Clark, G. M., Osborne, C. K., and Allred, D. C. Estrogen Receptor Status by Immunohistochemistry Is Superior to the Ligand-Binding Assay for Predicting Response to Adjuvant Endocrine Therapy in Breast Cancer. J.Clin.Oncol. 1999;17(5):1474-81.

167. Migliaccio, I., Wu, M. F., Gutierrez, C., Malorni, L., Mohsin, S. K., Allred, D. C., Hilsenbeck, S. G., Osborne, C. K., Weiss, H., and Lee, A. V. Nuclear IRS-1 Predicts Tamoxifen Response in Patients With Early Breast Cancer. Breast Cancer Res.Treat. 2010;123(3):651-60.

168. Allred, D. C., Harvey, J. M., Berardo, M., and Clark, G. M. Prognostic and Predictive Factors in Breast Cancer by Immunohistochemical Analysis. Mod.Pathol. 1998;11(2):155-68.

169. Kirkegaard, T., Edwards, J., Tovey, S., McGlynn, L. M., Krishna, S. N., Mukherjee, R., Tam, L., Munro, A. F., Dunne, B., and Bartlett, J.

M. Observer Variation in Immunohistochemical Analysis of Protein Expression, Time for a Change? Histopathology 2006;48(7):787-94.

170. Jensen, E. V., Block, G. E., Smith, S., Kyser, K., and DeSombre, E. R. Estrogen Receptors and Breast Cancer Response to Adrenalectomy. Natl.Cancer Inst.Monogr 1971;34:55-70.

171. Skidmore, J., Walpole, A. L., and Woodburn, J. Effect of Some Triphenylethylenes on Oestradiol Binding in Vitro to Macromolecules From Uterus and Anterior Pituitary. J.Endocrinol. 1972;52(2):289-98.

172. Jordan, V. C. Prolonged Antioestrogenic Activity of ICI 46, 474 in the Ovariectomized Mouse. J.Reprod.Fertil. 1975;42(2):251-8.

173. Jordan, V. C. and Dowse, L. J. Tamoxifen As an Anti-Tumour Agent: Effect on Oestrogen Binding. J.Endocrinol. 1976;68(02):297-303.

174. Jordan, V. C. and Koerner, S. Tamoxifen (ICI 46,474) and the Human Carcinoma 8S Oestrogen Receptor. Eur.J.Cancer 1975;11(3):205-6.

175. Effects of Chemotherapy and Hormonal Therapy for Early Breast Cancer on Recurrence and 15-Year Survival: an Overview of the Randomised Trials. Lancet 14-5-2005;365(9472):1687-717.

176. Hoos, A., Urist, M. J., Stojadinovic, A., Mastorides, S., Dudas, M. E., Leung, D. H., Kuo, D., Brennan, M. F., Lewis, J. J., and Cordon-Cardo, C. Validation of Tissue Microarrays for Immunohistochemical Profiling of Cancer Specimens Using the Example of Human Fibroblastic Tumors. Am.J.Pathol. 2001;158(4):1245-51.

177. Kononen, J., Bubendorf, L., Kallioniemi, A., Barlund, M., Schraml, P., Leighton, S., Torhorst, J., Mihatsch, M. J., Sauter, G., and Kallioniemi, O. P. Tissue Microarrays for High-Throughput Molecular Profiling of Tumor Specimens. Nat.Med. 1998;4(7):844-7.

178. McCarty, K. S., Jr., Miller, L. S., Cox, E. B., Konrath, J., and McCarty, K. S., Sr. Estrogen Receptor Analyses. Correlation of Biochemical and Immunohistochemical Methods Using Monoclonal Antireceptor Antibodies. Arch.Pathol.Lab Med. 1985;109(8):716-21.

179. Balmer, N. N., Richer, J. K., Spoelstra, N. S., Torkko, K. C., Lyle, P. L., and Singh, M. Steroid Receptor Coactivator AIB1 in Endometrial Carcinoma, Hyperplasia and Normal Endometrium: Correlation With Clinicopathologic Parameters and Biomarkers. Mod.Pathol. 2006;19(12):1593-605.

180. Chen, Y., Chen, C. F., Riley, D. J., Allred, D. C., Chen, P. L., Von Hoff, D., Osborne, C. K., and Lee, W. H. Aberrant Subcellular Localization of BRCA1 in Breast Cancer. Science 3-11-1995;270(5237):789-91.

181. Kurebayashi, J., Otsuki, T., Kunisue, H., Tanaka, K., Yamamoto, S., and Sonoo, H. Expression Levels of Estrogen Receptor-Alpha, Estrogen Receptor-Beta, Coactivators, and Corepressors in Breast Cancer. Clin.Cancer Res. 2000;6(2):512-8.

182. Fuqua, S. A., Schiff, R., Parra, I., Moore, J. T., Mohsin, S. K., Osborne, C. K., Clark, G. M., and Allred, D. C. Estrogen Receptor Beta Protein in Human Breast Cancer: Correlation With Clinical Tumor Parameters. Cancer Res. 15-5-2003;63(10):2434-9.

183. Harigopal, M., Heymann, J., Ghosh, S., Anagnostou, V., Camp, R. L., and Rimm, D. L. Estrogen Receptor Co-Activator (AIB1) Protein Expression by Automated Quantitative Analysis (AQUA) in a Breast Cancer Tissue Microarray and Association With Patient Outcome. Breast Cancer Res.Treat. 2009;115(1):77-85.

184. Wu, R. C., Qin, J., Hashimoto, Y., Wong, J., Xu, J., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. Regulation of SRC-3 (PCIP/ACTR/AIB-1/RAC-3/TRAM-1) Coactivator Activity by I Kappa B Kinase. Mol.Cell Biol. 2002;22(10):3549-61.

185. Yan, J., Yu, C. T., Ozen, M., Ittmann, M., Tsai, S. Y., and Tsai, M. J. Steroid Receptor Coactivator-3 and Activator Protein-1 Coordinately Regulate the Transcription of Components of the Insulin-Like Growth Factor/AKT Signaling Pathway. Cancer Res. 15-11-2006;66(22):11039-46.

186. Torres-Arzayus, M. I., Zhao, J., Bronson, R., and Brown, M. Estrogen-Dependent and Estrogen-Independent Mechanisms Contribute to AIB1-Mediated Tumor Formation. Cancer Res. 15-5-2010;70(10):4102-11.

187. Schneider, G., Reichert, M., Saur, D., Hamacher, R., Fritsch, R., and Schmid, R. M. HDAC3 Is Linked to Cell Cycle Machinery in MiaPaCa2 Cells by Regulating Transcription of Skp2. Cell Prolif. 2007;40(4):522-31.

188. Zhang, Y., Tuzova, M., Xiao, Z. X., Cruikshank, W. W., and Center, D. M. Pro-IL-16 Recruits Histone Deacetylase 3 to the Skp2 Core Promoter Through Interaction With Transcription Factor GABP. J.Immunol. 1-1-2008;180(1):402-8.

189. Huang, W., Tan, D., Wang, X., Han, S., Tan, J., Zhao, Y., Lu, J., and Huang, B. Histone Deacetylase 3 Represses P15(INK4b) and P21(WAF1/Cip1) Transcription by Interacting With Sp1. Biochem.Biophys.Res.Commun. 6-1-2006;339(1):165-71.

190. Chan, C. M., Lykkesfeldt, A. E., Parker, M. G., and Dowsett, M. Expression of Nuclear Receptor Interacting Proteins TIF-1, SUG-1, Receptor Interacting Protein 140, and Corepressor SMRT in Tamoxifen-Resistant Breast Cancer. Clin.Cancer Res. 1999;5(11):3460-7.

191. Chan, C. M., Martin, L. A., Johnston, S. R., Ali, S., and Dowsett, M. Molecular Changes Associated With the Acquisition of Oestrogen Hypersensitivity in MCF-7 Breast Cancer Cells on Long-Term Oestrogen Deprivation. J.Steroid Biochem.Mol.Biol. 2002;81(4-5):333-41.

192. Karmakar, S., Gao, T., Pace, M. C., Oesterreich, S., and Smith, C. L. Cooperative Activation of Cyclin D1 and Progesterone Receptor Gene Expression by the SRC-3 Coactivator and SMRT Corepressor. Mol.Endocrinol. 2010;24(6):1187-202.

193. Font, de Mora and Brown, M. AIB1 Is a Conduit for Kinase-Mediated Growth Factor Signaling to the Estrogen Receptor. Mol.Cell Biol. 2000;20(14):5041-7.

194. Lopez, G. N., Turck, C. W., Schaufele, F., Stallcup, M. R., and Kushner, P. J. Growth Factors Signal to Steroid Receptors Through Mitogen-Activated Protein Kinase Regulation of P160 Coactivator Activity. J.Biol.Chem. 22-6-2001;276(25):22177-82.

APPENDIX 1: FIGURES

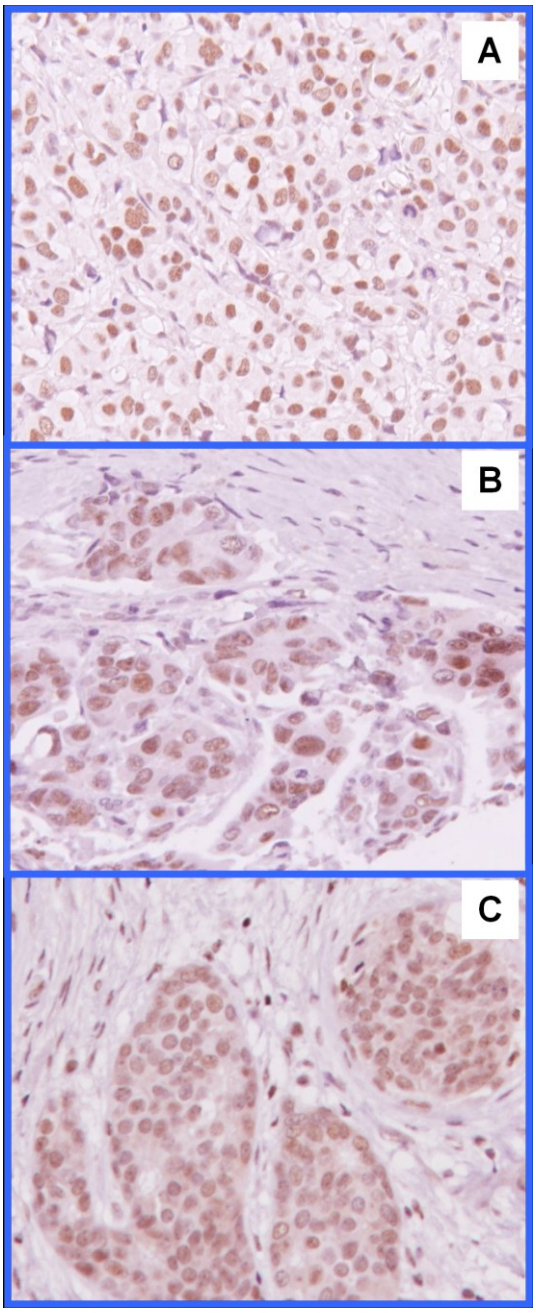

Figure 1: Representative images of invasive breast cancers stained for SRC1 (Panel A), SRC2 (Panel B) and AIB1 (Panel C).

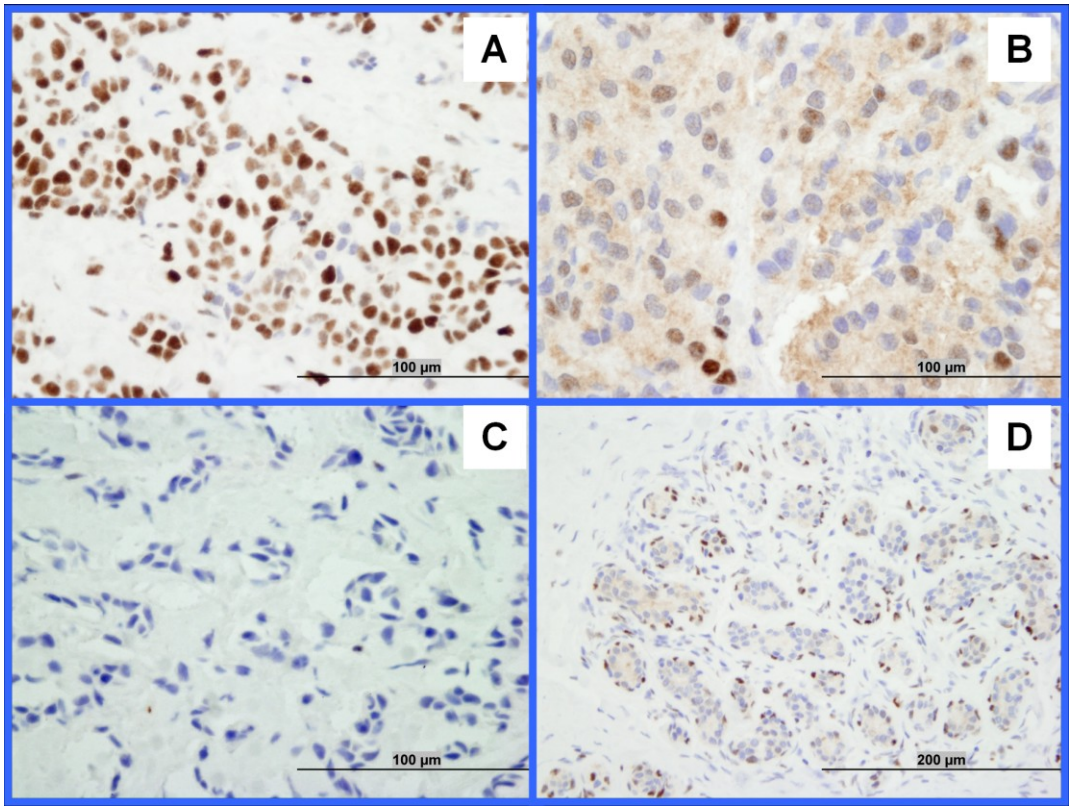

Figure 2: Representative images of invasive breast cancers (A-C) and normal TDLUs (D) stained for SMRT

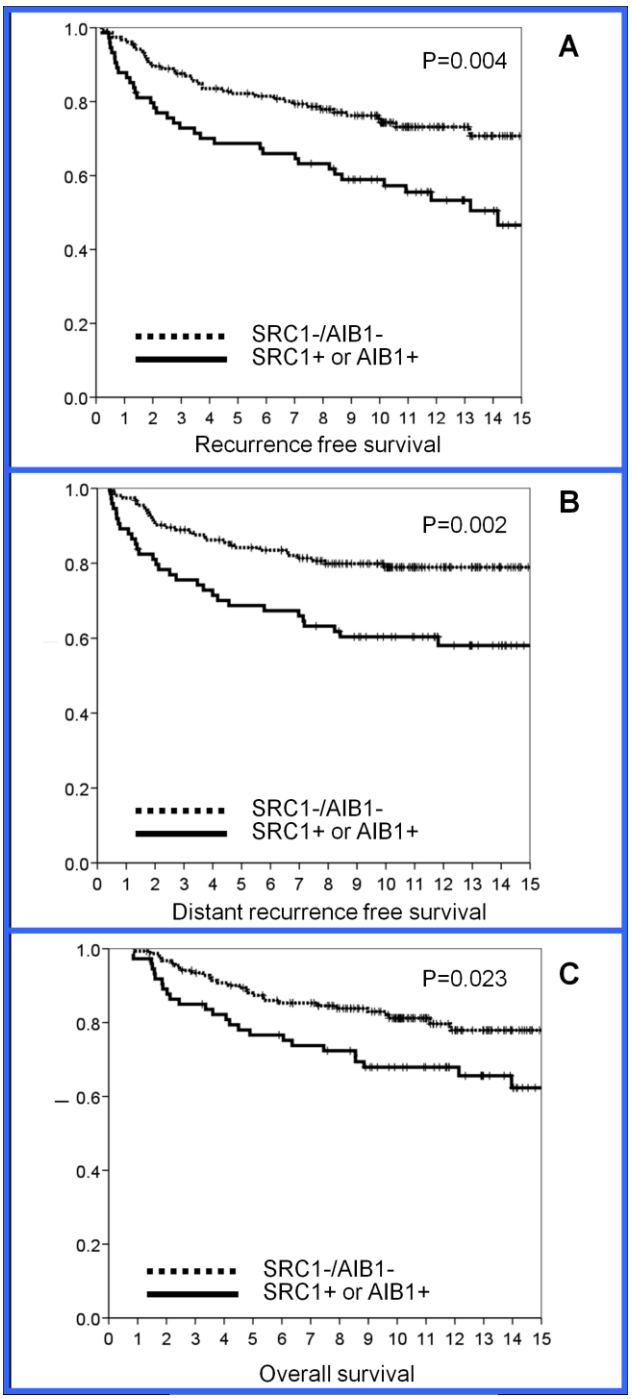

Figure 3: Kaplan-Meier survival curves of patients with ER negative invasive breast cancer overexpressing either SRC1 or SRC3 (solid lines) or negative for both SRC1 and SRC3 (dotted lines).

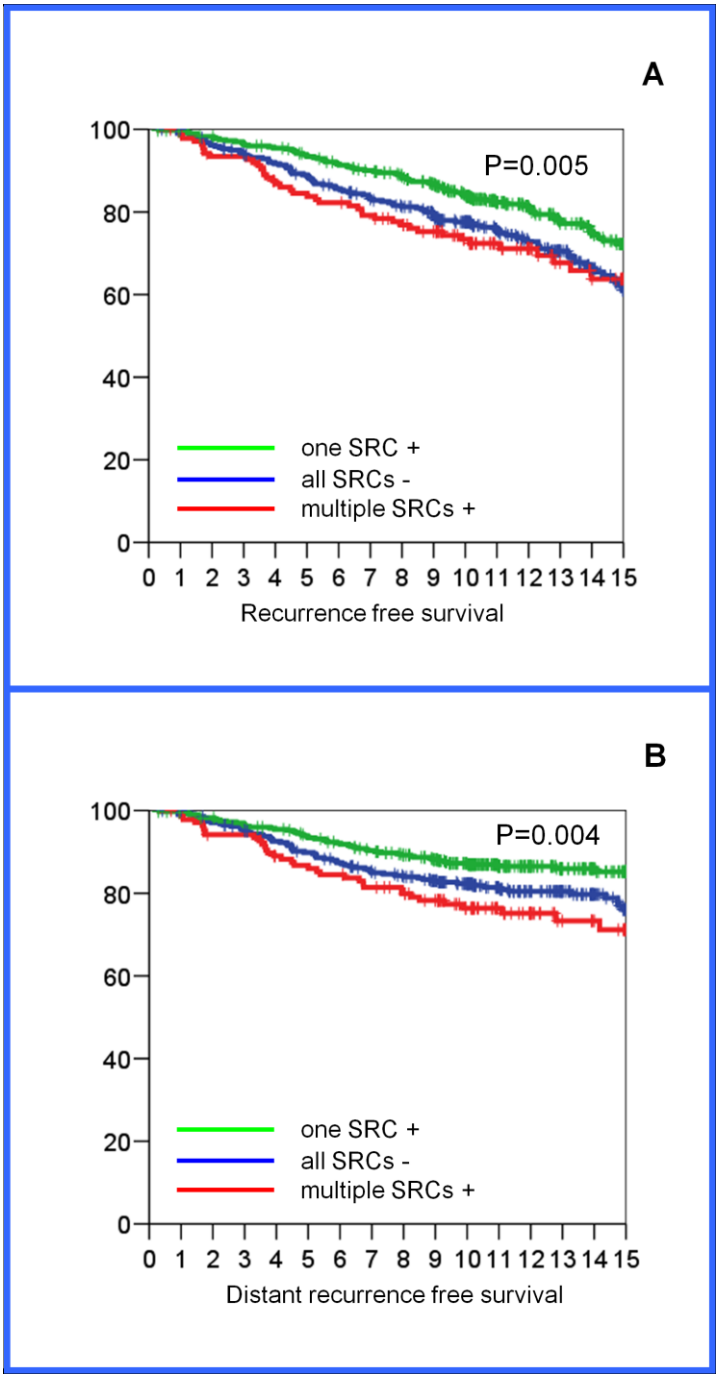

Figure 4: Kaplan-Meier survival curves of patients with ER positive tumors overexpressing either one SRC family member (either SRC1 *or* SRC2 *or* SRC3) (green lines), multiple SRC family members (red lines) or negative for all SRC family members (blue lines).

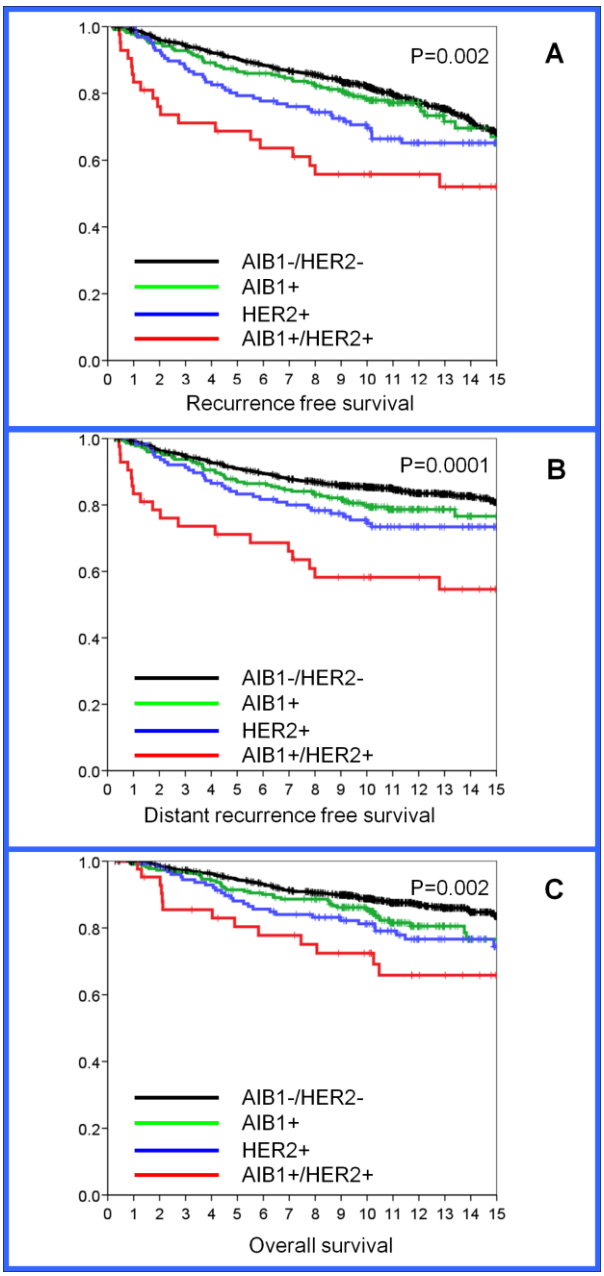

Figure 5: Kaplan-Meier survival curves of patients with tumors overexpressing either AIB1 (green lines), HER2 (blue lines) or both HER2/SRC3 (red lines) or negative for both HER2/SRC3 (black lines).

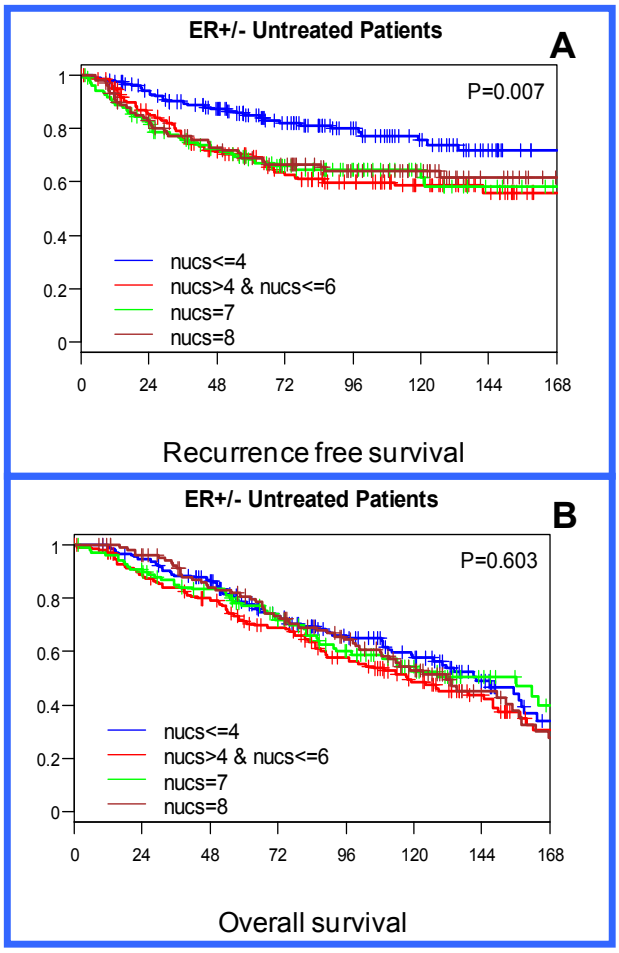

Figure 6: Kaplan-Meier survival curves of untreated patients with invasive breast cancer (both ER positive and ER negative) divided according to nuclear SMRT expression (1st quartile, blue lines; 2nd quartile, red lines, 3rd quartile, green lines; 4th quartile, purple lines)

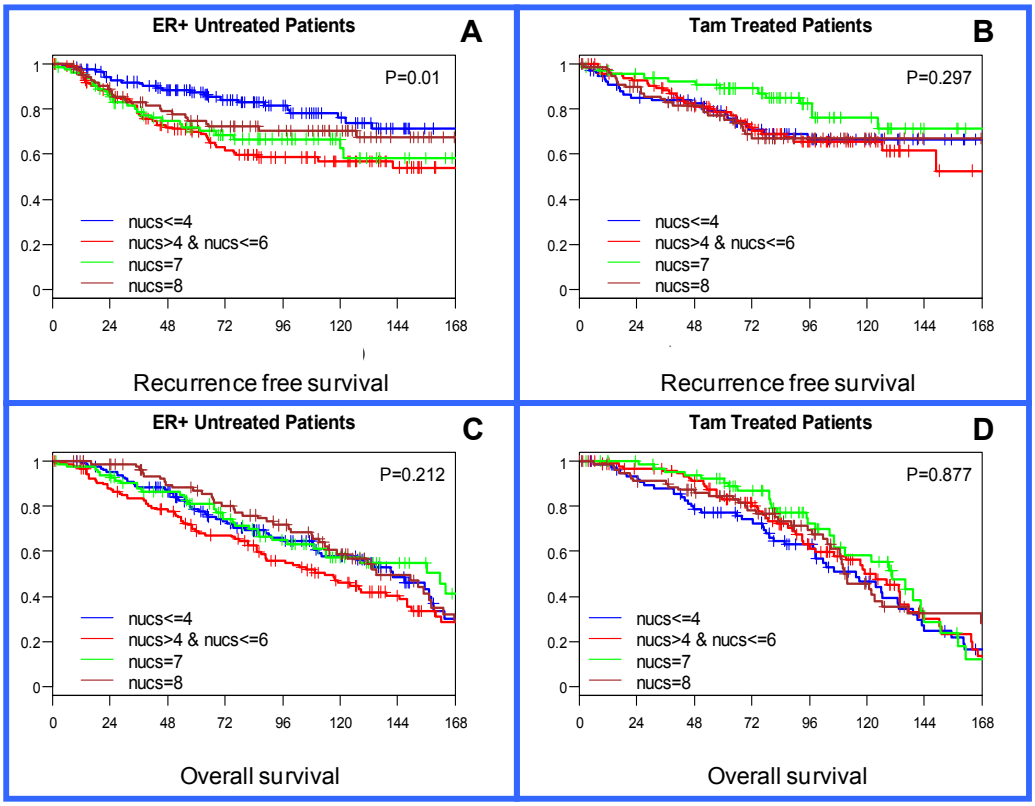

Figure 7: Kaplan-Meier survival curves of patients with ER positive invasive breast cancer divided according to treatment (untreated patients, panels A and C; tamoxifen monotherapy, panels B and D) and nuclear SMRT expression (1st quartile, blue lines; 2nd quartile, red lines, 3rd quartile, green lines; 4th quartile, purple lines)

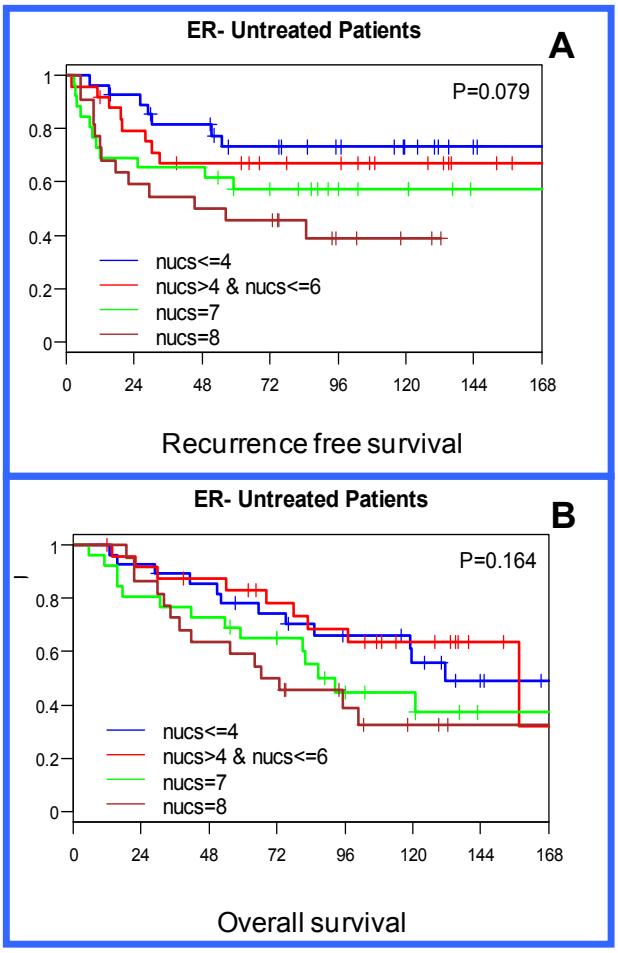

Figure 8: Kaplan-Meier survival curves of untreated patients with ER negative invasive breast cancer divided according to nuclear SMRT expression (1st quartile, blue lines; 2nd quartile, red lines, 3rd quartile, green lines; 4th quartile, purple lines)