Expression of the Forkhead Transcription Factor FOXP1 Is Associated with Estrogen Receptor α and Improved Survival in Primary Human Breast Carcinomas

Stephen B. Fox, Philip Brown, Cheng Han, Sally Ashe, Russel D. Leek, Adrian L. Harris, and Alison H. Banham

Nuffield Department of Clinical Laboratory Sciences, John Radcliffe Hospital, University of Oxford, Oxford, and Cancer Research UK Molecular Oncology Laboratory, Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, United Kingdom

ABSTRACT

Purpose: The FOXP1 protein belongs to a functionally diverse family of winged-helix or *forkhead* transcription factors that have diverse roles in cellular proliferation, differentiation, and neoplastic transformation. The *FOXP1* gene, which maps to 3p14, shows common loss of heterozygosity in breast tumors and is a candidate tumor suppressor gene. However, its role in breast cancer is unknown.

Experimental Design: We have therefore investigated the pattern of *FOXP1* expression in whole sections from normal (*n* = 16) and neoplastic (*n* = 90) breast tissues and correlated the level of expression in 283 invasive breast carcinomas on tissue microarrays with clinicopathological factors and survival. Because a relationship with estrogen receptor (ER) was identified, estrogen (17β-estradiol) regulation and ER/FOXP1 colocalization was also investigated.

Results: Expression of *FOXP1* was significantly positively associated with ER (*P* = 0.03) and negatively with epidermal growth factor receptor (170.01) but no association with age (*P* = 0.91), lymph node status (*P* = 0.94), size (*P* = 0.76), or grade (*P* = 0.22). In a multivariate analysis of survival, FOXP1 expression was associated with a significantly improved relapse-free (*P* = 0.03) and borderline overall (*P* = 0.09) survival. Unlike normal breast, there was common coexpression of FOXP1 and ER in cell lines and tumors, but no 17β-estradiol (10^-9 M) regulation of FOXP1 in MCF-7 cells was demonstrated.

Conclusions: Our findings support a role for FOXP1 as a potential ER coregulator in human breast carcinoma and suggest that it may also independently regulate additional important pathways that control the progression of breast cancer.

INTRODUCTION

There are many studies demonstrating genetic loss at the 3p locus in both *in situ* and invasive breast cancer and several regions, including 3p14, that might contain tumor suppressor genes have been identified (1–3). Candidate genes present at this site are fragile histidine triad, which spans the fragile site, FRA3B at 3p14.2 (4) and the recently identified transcription factor FOXP1 on 3p14.1 (5).

FOXP1 is a member of the winged helix or *forkhead* group of transcription factors that have diverse roles in cellular proliferation, differentiation, chromatin remodeling, mitotic program, and neoplastic transformation (reviewed in Ref. 6). Family members share a common DNA binding domain termed the winged-helix or *forkhead* domain with the FOXP subfamily having an additional NH2-terminal zinc finger motif. Murine Foxp1, which shares 97% homology with human FOXP1, has been shown to be a transcriptional repressor of lung-specific gene expression (7) and interleukin 2 expression (8). However, currently, little is known about the biological functions of the FOXP proteins or the identity of their target genes. We have recently shown that FOXP1 mRNA and protein are widely expressed in normal tissues, including lymph node, lung, brain, kidney, endocrine organs, reproductive system, and in skin, which is frequently altered in a wide range of tumors (5). We have further shown variable expression of FOXP1 protein in a pilot series of *in situ* and invasive breast tumors (5).

In view of a possible role of FOXP1 as a tumor suppressor gene in the development of breast cancer, we have extended our studies and examined the expression of FOXP1 in normal breast tissue and *in situ* and invasive carcinomas. Our aims were to determine the pattern of FOXP1 protein in different stages of breast cancer progression, examine the relationship of FOXP1 expression with clinicopathological factors, and assess whether its expression impacts on patient survival. These studies identified a significant association with expression of estrogen receptor (ER) that were additionally investigated.

MATERIALS AND METHODS

Patients and Tumors

Whole tissue sections from breast carcinomas (*n* = 90) and histologically normal breast tissues (*n* = 16) together with microarrayed tissues from 283 breast carcinomas (including the 90 tumors studied in whole sections) were collected from patients undergoing surgery at The John Radcliffe Hospital (Oxford, United Kingdom) with approval from the Local Ethics committee (C02.216). Tumors were treated by mastectomy (*n* = 100).
Table 1  Correlation analyses between FOXP1 and clinicopathological, angiogenic, and hypoxic data for 283 invasive breast carcinomas studied by tissue microarray

<table>
<thead>
<tr>
<th>Total no. of patient</th>
<th>FOXP1 Negative</th>
<th>FOXP1 Positive</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>Age (yrs)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;50</td>
<td>41</td>
<td>41</td>
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</tr>
<tr>
<td>≥50</td>
<td>99</td>
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<td>77</td>
<td>78</td>
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</tr>
<tr>
<td>Positive</td>
<td>63</td>
<td>65</td>
<td></td>
</tr>
<tr>
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<tr>
<td>≤2 cm</td>
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<td>0.76</td>
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<tr>
<td>&gt;2 cm</td>
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<td>68</td>
<td></td>
</tr>
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<td>Grade</td>
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<td>I</td>
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<td>34</td>
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</tr>
<tr>
<td>III</td>
<td>43</td>
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<td></td>
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<td>66</td>
<td>0.01a</td>
</tr>
<tr>
<td>Positive</td>
<td>95</td>
<td>74</td>
<td></td>
</tr>
</tbody>
</table>

a Denotes significance.

70) or lumpectomy (n = 213), axillary node sampling with node status confirmed histologically. Primary histological types included 204 ductal carcinomas of no specific type, 18 lobular, and 20 others. Grading of was performed according to the modified Bloom and Richardson method (9). The clinicopathological characteristics of the series are presented in Table 1. In patients <50 years, adjuvant cyclophosphamide, methotrexate, and 5-fluorouracil was administered if tumors were node positive, or ER negative and/or ≥3 cm. Patients <50 years with ER negative, node-positive tumors also received cyclophosphamide, methotrexate, and 5-fluorouracil. The median follow up was 7.3 years (range, 0.2–11.3 years) in which there were 100 relapses and 71 deaths.

FOXP1 Immunohistochemistry

Four-μm formalin-fixed, paraffin-embedded sections of normal and primary breast tumors were immunostained with the mouse monoclonal antibody JC12 as previously described using the EnVision Detection kit, peroxidase/3,3’-diaminobenzidine (DAKO, Glostrup, Denmark; Ref. 5). JC12 has been shown to react specifically with FOXP1 and not with the closely related proteins FOXP2, FOXP3, and FOXP4 (data not shown). The pattern of FOXP1 expression was determined from whole tissue sections, and the level of neoplastic cell nuclear expression from tissue microarrays was scored using the following system: negative = 0; weak/focal staining = 1; strong focal/widespread moderate staining = 2; or strong/widespread staining = 3. Grade 2 and 3 tumors were considered positive for FOXP1 in statistical analyses.

Cytospin preparations of ER-positive (MCF-7, T47D and ZR751) and ER-negative (MDA-MB-231, MDA-MB-468) breast cancer cell lines [all from American Type Culture Collection (Manassas, VA)] were fixed in ice-cold methanol for 10 min and immunostained using antibody JC12 diluted 1:10 in PBS/10% normal human serum, followed by detection with EnVision (DAKO).

Double Immunofluorescent Labeling of FOXP1 and ER in Breast Cancer Cell Lines and Tissues

Cytospin preparations of the MCF-7 breast cancer cell line were prepared and fixed as described above then blocked with Serum-free Protein Block (DAKO) for 10 min, rinsed in PBS, then 1:50 dilutions of the primary antibodies JC12 (IgG2a) and 1D5 (IgG1, anti-ERα; DAKO) were applied together for 1 h at room temperature. After washing in PBS, the isotype-specific secondary antibodies, goat antimouse IgG2a AlexaFluor-488, and goat antirabbit IgG, AlexaFluor-568 (Molecular Probes, Eugene, OR) were added at 30 μg ml−1 and incubated for an additional 1 h at room temperature. The slides were rinsed thoroughly in PBS and coverslips applied with a few drops of Fluorescent Mounting Medium (DAKO). The isotype specificity of secondary antibodies was confirmed by labeling cells with JC12 and 1D5 singly, followed by both secondary antibodies. In each case, only specific single color labeling was detected.

For normal and neoplastic breast tissues, antigen retrieval was performed as described above and reagents applied for the MCF-7 cell line, except that the JC12 antibody was used at 1:10 dilution.

In Vitro Estrogen Regulation Experiments

Cytospins.  MCF-7 cells were grown to 75% confluence in RPMI 1640 (Sigma) supplemented with 10% FCS, 2 mM L-glutamine, 50 units ml−1 penicillin, and 50 μg ml−1 streptomycin (complete medium; Invitrogen), at 37°C, in 5% CO2. Cells were washed thoroughly in PBS and then incubated in phenol red-free RPMI 1640 (Invitrogen), 10% charcoal-stripped FCS (Sigma) 50 units ml−1 penicillin, and 50 μg ml−1 streptomycin (Invitrogen) for an additional 72 h before being treated with either 10−9 M 17β-estradiol (E2; Sigma), 10−6 M 4-hydroxytamoxifen (Sigma), both of these or neither (vehicle only), for an additional 16 h. The medium was decanted and retained, and the cells were harvested in PBS/0.53 mM EDTA, pelleted, and resuspended in their original culture medium. Cytocentrifuge preparations of the cells were fixed for 10 min in ice-cold methanol, then immunostained either with JC12 (1:10), pS2 (1:50, DAKO) or diluent alone (10% normal human serum in PBS) as described above. The experiment was performed in triplicate, and the proportion of cells staining with JC12 and pS2 were then counted for each sample. The pS2 expression was used to confirm estrogen induction of a known ER target in the appropriate sample.

Western Blotting.  Nuclear proteins from the control and treated MCF-7 cells described above were extracted using the NE-PER extraction reagents (Pierce) as described in the supplier’s protocol. Proteins were solubilized in 1× SDS loading buffer [50 mM Tris (pH 6.8), 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 5% (v/v) glycerol, and 100 mM DTT] and resolved in 10% acrylamide gels in 1× SDS running buffer [25 mM Tris, 250 mM glycine, and 0.1% (w/v) SDS]. After separation, proteins were transferred to polyvinylidene fluoride membrane (Immobilon-P; Millipore) in transfer buffer (10% metha-
Fig. 1  FOXP1 expression in normal and neoplastic breast tissues. FOXP1 expression in the nuclei of myoepithelial cells alone (A) and in myoepithelial and ductal cells of the ducts and acini of the terminal ducts lobular unit (inset, estrogen receptor α; B). Strong FOXP1 immunostaining in epithelium showing apocrine metaplasia in the nuclear and cytoplasmic compartments (C). Strong pan-tumor nuclear FOXP1 positivity in situ and invasive breast carcinoma of no specific type (NST; inset shows cytoplasmic only staining for FOXP1; D), which was observed in some cases accentuated at the invasive tumor edge (arrows; E). Strong expression of nuclear FOXP1 in an invasive lobular carcinoma, which has a targetoid pattern surrounding entrapped normal duct elements (asterisk; F). Heterogeneous nuclear expression in an invasive ductal carcinoma, NST (G). Only occasional nuclear expression of FOXP1 in a tumor with adjacent necrosis (asterisk; H). An invasive ductal carcinoma, NST, which is negative for FOXP1 adjacent to FOXP1-positive normal acinar elements (I). Nonneoplastic stromal cells, both matrix fibroblasts (short arrows) and inflammatory cells (long arrows), also expressed FOXP1 (J). FOXP1 expression in the nuclei of endothelial cells lining small capillaries (arrows) surrounding a FOXP1-positive invasive ductal carcinoma, NST (K). A tumor-associated microvessel demonstrating heterogeneous nuclear endothelial and pericyte immunopositivity (L). Entrapped arterioles were also occasionally positive for FOXP1 both in endothelial cells and surrounding smooth muscle cells of the media and adventitia (M).
Fig. 2  A, confocal microscope photomicrograph montage of MCF-7 breast cancer cell lines double immunolabeled with JC12 (FOXP1, red) and 1D5 [estrogen receptor (ER), green] with colocalization of both in yellow. Top three panels are low power photomicrographs showing both independent nuclear ER and FOXP1 staining but also double staining of both nuclear proteins in some MCF-7 cells. The bottom three panels are high-power photomicrographs of a breast cancer cell from the above area (arrow) showing colocalization of FOXP1 and ER in the nucleus. B, confocal microscope
RESULTS

Expression of FOXP1 Protein in Normal Breast Tissues and Breast Carcinomas.

In normal breast tissues, different patterns of epithelial immunoreactivity were identified. Some small ducts and acini of the terminal duct lobular unit showed homogeneous nuclear expression in myoepithelial cells alone (Fig. 1A), whereas in other units, expression was in both myoepithelial and inner ductal cells (Fig. 1B); occasional stromal cells were also positive for FOXP1. FOXP1 was additionally expressed strongly in larger ducts and in areas of epithelial cell hyperplasia. FOXP1 was present almost exclusively in the nucleus in nonneoplastic tissues apart from in areas demonstrating apocrine metaplasia where both cytoplasmic and nuclear expression was observed (Fig. 1C). FOXP1 was expressed in invasive tumors (Fig. 1D) with occasional cases showing up-regulation at the invasive edge (Fig. 1E). Although FOXP1 was identified in all histotumor types examined (Fig. 1F), expression in both in situ and invasive tumors was usually variable in the proportion of cells staining and their intensity of expression (Fig. 1G). FOXP1 was mostly expressed in the nuclear compartment but cytoplasmic reactivity was also frequently identified (40 of 90 tumors; Fig. 1H). No enhancement of staining was observed adjacent to areas of necrosis (Fig. 1H). Tumors showing loss of FOXP1 were confirmed because normal entrapped epithelial elements remained immunopositive (Fig. 1I). FOXP1 was not confined to the epithelial elements in tumors but was also present in stromal elements (Fig. 1J), including fibroblasts, inflammatory cells, and in vessels both in endothelia and pericytes. For the latter, FOXP1 was seen in all types and calibers of vessels (Fig. 1, K and L). In tissue microarrays, 43 cases were scored 0, 97 scored 1, 85 scored 2, and 58 scored 3. The cutoff of 0, 1 versus 2, 3 split patients into approximately equal-sized groups of 140 negative and 143 positive. There was a significant correlation between the scores derived whole tissue and array sections (P < 0.001).

Relationship between FOXP1 Expression and Clinicopathological Variables and Survival.

There was a significant positive correlation between FOXP1 and ER (P = 0.03) and inverse association with epidermal growth factor receptor (P = 0.01). There was no correlation between FOXP1 and patient age (P = 0.91), lymph node status (P = 0.94), tumor size (P = 0.76), or tumor grade (P = 0.22; Table 1). In the univariate analysis, patients with FOXP1-positive tumors showed longer relapse-free and overall survival, although this was only significant for relapse-free survival (P = 0.05 and P = 0.14, respectively). Because this may be due to its strong association with ER, we also performed a multivariate analysis. This confirmed that FOXP1 was independent with a significant reduction in relapse-free survival (P = 0.03) and borderline in overall survival (P = 0.09), which for power, was a similar order of magnitude as tumor grade (Table 2).

Confocal Microscopy.

To further characterize the relationship identified between the expression of FOXP1 and ER, FOXP1 expression was investigated in three ER-positive (MCF-7, T47D, and ZR751) and two ER-negative breast cancer cell lines (MDA-MB-231 and MDA-MB-468). All of the cell lines expressed nuclear FOXP1 protein, and although both the level of expression and the proportion of positive cells varied (data not shown), colocalization of the FOXP1 and ER proteins in MCF-7 breast cancer cell nuclei was observed (Fig. 2A).

To examine for potential differences of colocalization be-

nol, 1× SDS running buffer). Membranes were then incubated in blocking buffer [5% (w/v) Marvel, 1× PBS] for 1 h and then incubated in blocking buffer with the indicated primary mouse monoclonal antibody for 90 min at room temperature. Membranes were washed for 30 min in three changes of wash buffer [1× PBS, 0.05% (v/v) Tween] and then incubated with the horseradish peroxidase-conjugated goat antimouse secondary antibody (DAKO) in blocking buffer for 90 min at room temperature. Membranes were washed as before and proteins detected using the enhanced chemiluminescence reagent (Amersham). The anti-FOXP1 monoclonal antibody (JC12) and anti-nucleophosmin antibody (NA24; Ref. 10) loading control were both used at a 1 in 30 dilution. The horseradish peroxidase-conjugated goat antimouse antibody was used at a 1 in 1500 dilution.

Statistical Analysis. The χ² test was used to test for differences between categorical variables and the log-rank test for differences in survival with the Cox proportional hazard model for independence. All tests were performed using Stata 7.0 (Stata Corporation, College Station, TX).

Table 2 Cox proportional hazard model for relapse-free and overall survival, including FOXP1 expression with other standard clinicopathological factors

<table>
<thead>
<tr>
<th>Hazard ratio</th>
<th>95% confidence interval</th>
<th>P</th>
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<tr>
<td>Relapse-free survival</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nodes</td>
<td>2.94</td>
<td>1.74–4.96</td>
<td>0.000</td>
</tr>
<tr>
<td>Size</td>
<td>3.39</td>
<td>1.98–5.83</td>
<td>0.000</td>
</tr>
<tr>
<td>Grade</td>
<td>1.67</td>
<td>1.16–2.40</td>
<td>0.006</td>
</tr>
<tr>
<td>FOXP1</td>
<td>0.61</td>
<td>0.38–0.98</td>
<td>0.04</td>
</tr>
<tr>
<td>Overall survival</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nodes</td>
<td>3.20</td>
<td>1.70–6.03</td>
<td>0.000</td>
</tr>
<tr>
<td>Size</td>
<td>4.56</td>
<td>2.26–9.19</td>
<td>0.000</td>
</tr>
<tr>
<td>Grade</td>
<td>2.08</td>
<td>1.32–3.38</td>
<td>0.001</td>
</tr>
<tr>
<td>FOXP1</td>
<td>0.62</td>
<td>0.36–1.07</td>
<td>0.09</td>
</tr>
</tbody>
</table>

- Table 2: Cox proportional hazard model for relapse-free and overall survival, including FOXP1 expression with other standard clinicopathological factors.
FOXP1 in Breast Cancer

Estrogen Regulation of FOXP1 Expression in the MCF-7 Breast Cancer Cell Line. MCF-7 cells were grown in charcoal-stripped medium with the addition of estrogen (E2), tamoxifen, both treatments, or ethanol (vehicle only) as a control. Immunohistochemistry was then performed to confirm estrogen regulation of pS2 expression. A clear up-regulation of pS2 expression in the E2 treated cells was observed, demonstrating that the E2 treatment was effective (Fig. 3). Western blotting experiments using nuclear extracts prepared from the remaining cells from this experiment were used to quantify FOXP1 expression levels. These showed two dominant FOXP1 bands, one at 60 kD, one at 37 kD, and one weak band, probably reflecting the different splice variants, that were unaffected by any of the treatments used when taking into account the loading control (Fig. 3).

DISCUSSION

We have used a monoclonal antibody that identifies FOXP1 but not its close relatives FOXP2, FOXP3, or FOXP4 to examine its expression in normal breast tissue and in situ and invasive breast carcinomas. FOXP1 is present in normal tissues of varying patterns in epithelial elements of the terminal duct lobular unit and in stromal cells, suggesting a physiological role in normal breast cyclical changes. We observed two common expression patterns, myoepithelial alone and myoepithelial and ductal expression, both patterns also showing occasional stromal cell expression. Changes in myoepithelial cell morphology are known to occur through the menstrual cycle, organizing milk production. In ER-positive tumors, most neoplastic epithelial cells showed coexpression of FOXP1 and ER with only few cells demonstrating sole expression of either FOXP1 or ER (Fig. 2B). Thus, although occasional colocalization was identified in normal human breast tissues, this was more frequent in breast carcinomas.

In breast tumors, the expression of FOXP1 was found to correlate significantly with that of ER-α. One explanation for this relationship was the possibility that the expression of FOXP1 might be regulated by estrogen. Because many nuclear receptors shuttle to and from the nucleus, in our hormone response experiments, we examined FOXP1 protein expression using both immunohistochemical labeling of cytospins and Western blotting of nuclear extracts to investigate potential changes in both expression levels and subcellular localization. No change in either the nuclear distribution of FOXP1 (data not shown) or the quantity of nuclear FOXP1 protein was observed in the ER-positive MCF-7 cells on E2 administration.

Another alternative is that FOXP1 might act as a coregulator of the ER, the lack of E2 regulation is consistent with data obtained for other known ER coregulatory molecules whose levels are also unaffected by E2 (13). Ligand-dependent activation of gene transcription by nuclear receptors such as retinoic acid receptor, thyroid hormone receptor, ER, and peroxisome proliferator-activated receptor γ are dependent on the recruitment of coactivators. The α-helical LXXLL motif found in some coactivators is sufficient for ligand-dependent interaction with nuclear receptors and this signature motif, commonly referred to as the nuclear receptor box, is present in the NH2 terminus of the FOXP1 protein, raising the possibility that FOXP1 might physically associate with the ER. Additional amino acids flanking the nuclear receptor box (e.g., positions -1 and +6) and the identity of the amino acids at +2 and +3 are also important for full activation.

...
(XX) are important for coactivator choice by nuclear receptors (14). The FOXP1 protein differs from FOXP2, FOXP3, and FOXP4 at position +3, whereas the FOXP3 protein differs from FOXP1, FOXP2, and FOXP4 at positions −1 and +6, raising the possibility that these may reflect a functional difference in receptor binding between the different FOXP proteins.

Double-labeling studies have confirmed colocalization of FOXP1 and the ER, supporting the notion that FOXP1 may act as an ER coregulator. Indeed, other forkhead proteins from the FOXO family such as FKHR and the related proteins, FKHL1 and AFX, which also have a conserved LXXLL motif, also interact with ER-α (15, 16).

In mice, Foxp1 acts as a transcriptional repressor (7), and splice variants are generated that can homo- and heterodimerize with other members of the Foxp family, which alters the repressive activity (8). Similarly, FOXP1 splice variants that may have different biological functions have already been identified. In support of this hypothesis is the identification a splice variant of FOXP1 (cDNA, pAB196) that lacks the NH2-terminal coiled-coil domain and most of the second glutamine-rich domain. Importantly, this variant has also lost the LXXLL motif (5) that may mediate its binding to the ER (17). Indeed, Western blotting using the JC12 antibody identified up to three different variants (cDNA, pAB196) that lacks the NH2-terminal coiled-coil domain and most of the second glutamine-rich domain. Importantly, this variant has also lost the LXXLL motif (5) that may mediate its binding to the ER (17). Indeed, Western blotting using the JC12 antibody identified up to three different molecular weight proteins in MCF-7 breast cancer cells that may be the products of such splice variants. There are numerous examples of proteins with differing biological activities generated from a single gene by alternative splicing and extensive alternative splicing has also been reported for the FOXP2 gene (18). Moreover, the expression of alternatively spliced FOXP1 proteins may explain the presence of cytoplasmic staining that was observed in some tumors that was not identified in normal breast tissues.

The genes transcriptionally regulated by FOXP1 are largely unknown, but in mice, Foxp1 targets appear to include some tissue-restricted genes (7, 8). In the breast, the major target may be those genes regulated by ER and a potential functional association between FOXP1, and the ER may account for the effect we observed on survival. Furthermore, because FOXP1 also appears to have an independent effect for at least relapse-free survival, it also suggests that FOXP1 functions in other pathways, and this transcription factor is likely to bind target promoters and regulate gene expression independently of the ER.

Additional work assessing the role of FOXP1 as a potential coregulatory molecule of ER is warranted, and future studies to confirm the physical interaction of these proteins and to identify the domains involved and the affects on ER-mediated gene expression will be undertaken in our laboratory. Because other ER coregulatory molecules have been implicated in endocrine resistance, it would also be of interest to assess whether FOXP1 gives any predictive information as to response to such treatments (19, 20). Furthermore, in view of the close relationship in tumors between FOXP1 and ER-α, it will be important also to assess the role of the more recently identified ER-β. Interestingly, FOXP1 is expressed in both myoepithelial and stromal cells, a similar pattern to that of ER-β (21) in normal breast tissue.

REFERENCES

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