Expression of AP-2 Transcription Factors in Human Breast Cancer Correlates with the Regulation of Multiple Growth Factor Signalling Pathways

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ABSTRACT

The AP-2 transcription factors are required for normal growth and morphogenesis during mammalian development. Previous in vitro studies have also indicated that the AP-2 family of proteins may be involved in the etiology of human breast cancer. The AP-2 genes are expressed in many human breast cancer cell lines, and critical AP-2-binding sites are present in both the ERBB-2 (HER2/neu) and estrogen receptor promoters. We have now characterized immunological reagents that enable specific AP-2 family members, including AP-2α and AP-2γ, to be detected in human breast cancer epithelium. Data obtained with these reagents demonstrate that whereas AP-2α and AP-2γ are both present in benign breast epithelium, there is a significant up-regulation of AP-2γ expression in breast cancer specimens (P = 0.01). There was also a significant correlation between the presence of the AP-2α protein and estrogen receptor expression (P = 0.018) and between specimens containing both AP-2α/AP-2γ proteins and ERBB-2 expression (P = 0.003). Furthermore, we detected an association (P = 0.04) between the expression of AP-2γ and the presence of an additional signal transduction molecule implicated in breast cancer, the insulin-like growth factor I receptor. Analysis of the proximal promoter of the insulin-like growth factor I receptor revealed a novel AP-2-binding site.Thus, AP-2 proteins may directly regulate the expression of this growth factor receptor. Taken together, these data strongly support a role for the AP-2 gene family in the control of cell growth and differentiation in breast cancer.

INTRODUCTION

Recent advances in molecular genetics have provided new insights into the etiology of human breast cancer. Whereas mutations in the BRCA1 and BRCA2 genes have been identified in some patients with familial breast cancer, other molecular markers may help to classify sporadic breast tumors, which account for approximately 90% of newly diagnosed breast cancers (1–5). In particular, the expression status of two critical signal transduction molecules, ERBB-2 (HER2/neu) and the ER, has important implications for patient prognosis, appropriate adjuvant treatment, and treatment response (1, 2, 6–8). Insight into the regulatory networks controlling the expression of these important signal transduction pathways in breast cancer may yield new gene targets for prognosis and therapeutic intervention.

In normal mammary gland development, ER molecules are important for signaling ductal proliferation during puberty and pregnancy. The ER gene is also expressed at elevated levels in approximately 50% of all breast carcinomas (6, 8, 9). Patients with ER-positive breast carcinomas have an improved survival rate and a longer disease-free interval than patients whose tumors lack ER expression because the ER-positive tumors are usually hormone sensitive, and their growth can be controlled with hormonal therapy. ERBB-2 overexpression is also a common molecular alteration observed in human breast cancer, in which it occurs in approximately 15% of breast tumors (2). Moreover, high levels of ERBB-2 have been shown to correlate with poor prognosis and to predict a worse response to chemotherapy, mastectomies, and breast-conserving therapy (2, 7). The oncogenic potential of ERBB-2 has also been demonstrated in both cultured cells and transgenic mouse models, strongly suggesting that this protein has a role in the pathogenesis of human breast cancer (2).

The AP-2 gene family of transcription factors has been implicated in regulating both ER and ERBB-2 expression during the progression from normal breast epithelium to breast cancer (10–12). All three known members of this gene family, AP-2α, AP-2β, and AP-2γ, are expressed in human breast cancer cell lines (11). During the normal course of vertebrate embryogenesis, these genes are transcribed in tissues undergoing complex morphogenetic changes. Major sites of AP-2 expression include the developing neural crest, kidney, epidermis, and face and limb buds (13–15). Thus far, two of these genes have been disrupted by homologous recombination. AP-2α knockout mice have multiple defects in the development of the neural tube, face, eyes, limbs, and body wall (16, 17). Mice lacking the AP-2β gene have fewer gross phenotypic defects but nevertheless die shortly after birth due to a failure of collecting duct formation in the kidneys (18). Together, these findings establish the importance of the AP-2 gene family for normal growth and morphogenesis during embryonic development.

The three related AP-2 proteins all bind as homo- or heterodimers to a GC-rich element typified by the DNA sequence GCCNNNGGC-3', occurs in the 5' untranslated region of the ER gene and augments ER promoter activity in vitro (12, 20). In the context of the ERBB-2 promoter, our previous studies have shown that the sequence element 5'-GCTTCACAGGC-3', which is required for high-level expression of this gene in human breast cancer cell lines, also acts as an AP-2-binding site (10). Moreover, we have demonstrated that the AP-2 proteins can act through this binding site to regulate ERBB-2 promoter activity (11). Further support for the role of the AP-2 proteins in ERBB-2 overexpression came from an examination of breast cancer cell lines. In these in vitro experiments, high levels of ERBB-2 expression correlated with the presence of one or more AP-2 proteins, especially that of AP-2α and AP-2γ (10, 11). No ERBB-2 overexpression was found in the absence of AP-2, although AP-2 expression...
could be detected in some cell lines that lacked ERBB-2 transcription (21).

The presence of AP-2 proteins in several breast cancer cell lines raised the possibility that an in vivo analysis of this gene family could provide mechanistic insight into the etiology of mammary tumors. Therefore, we have now examined the incidence of AP-2 protein expression in vivo, both in BBE and breast cancer specimens, to determine whether expression of the AP-2 proteins was up-regulated during the progression toward breast cancer. We have also extended these studies to determine whether the presence of the AP-2a and AP-2-y transcription factors in human breast cancer specimens correlated with the expression of candidate target genes, especially that of growth factor receptor molecules. As outlined below, our findings strongly support an important role for AP-2 in the etiology of human breast cancer.

MATERIALS AND METHODS

Patient Population and Clinical Characteristics. The breast cancer database has 2400 patients treated between 1973 and 1993 at Yale University School of Medicine with a median follow-up of 13 years and a minimum follow-up of 4 years. From this group of patients, we identified 81 patients who had experienced early-stage breast cancer and were treated with lumpectomy and radiation therapy whose archival paraffin-embedded tumor blocks were available. All patients were treated by lumpectomy, with or without axillary dissection, followed by full-course radiation therapy to the intact breast to a median dose of 64 Gy. The age, tumor histology, tumor size, stage distribution, axillary lymph node status, date of radiation therapy treatment, menopausal status, type of adjuvant systemic treatment, length of follow-up, DDFS, and OS were available on all patients in the study (22). All patients were seen in follow-up every 6 months for the first 3 years and yearly thereafter. The 81 early-stage breast cancer patients presented at a mean age of 53 years and had a median follow-up of 10 years. The 10-year DDFS and OS for the patient population in this study are 60 and 73%, respectively. The majority of the patients had a histological diagnosis of IDC (73 of 81 patients) or invasive lobular carcinoma (5 of 81 patients), and some patients had invasive medullary carcinoma (3 of 81 patients). All tumors specimens were derived from patients with $T_1$ (tumor < 2 cm) or $T_2$ (tumor = 2–5 cm) breast tumors, and there was no bias toward either small or large breast tumors in this study. Note that although 81 total samples were used in the study, there was not always enough tissue available to use for all probes. In particular, 80 samples were processed for AP-2a expression, of which 74 were also analyzed for the presence of the AP-2-y protein. One sample was analyzed for AP-2-y but not AP-2a expression. With respect to ERBB-2, 76 samples were analyzed; 75 were assessed for AP-2a status, 70 were assessed for AP-2-y status, and 69 were assessed for both AP-2a and AP-2-y status. We were also able to obtain the paraffin-embedded blocks of 12 women who underwent reduction mammoplasties in which a pathological analysis revealed no evidence of malignancy. A protocol for the study was approved by the Human Investigations Committee at the Yale University School of Medicine.

Immunohistochemistry. After the identification of both benign and invasive breast cancer cases, the individual 10% formalin-fixed and paraffin-embedded blocks were evaluated for either BBE or invasive carcinoma by H&E staining and processed for immunohistochemical staining as described previously (22). The 5-μm-thick sections were deparaffinized in xylene and washed in ethanol before treatment with hydrogen peroxide to quench endogenous peroxidase. After rehydration, slides used for AP-2a immunohistochemistry were pressure-cooked for 5 min in 9 mM sodium citrate (pH 6.0). Slides used for AP-2-y staining or staining with the SC-184 polyclonal antiserum (Santa Cruz Biotechnology, Santa Cruz, CA) did not require antigen retrieval. Subsequently, all sections were washed in 1% PBS and blocked with goat suppressor serum for 30 min. Next, immunological reagents capable of distinguishing between the three AP-2 gene products were added overnight at 4°C. AP-2a status was determined by using the AP-2a-specific mouse monoclonal antibodies 3B5 or 5E4 (11, 17), either undiluted from tissue culture supernatant or as a 1:1000 dilution of ascites fluid. The 3B5 antibody recognizes an epitope in the DNA-binding domain of AP-2a, and an AP-2a peptide, GINIPDQTVKKGPSLSDKSSQVSAIPINK (23), can act as a specific blocking reagent. AP-2-y status was analyzed using a 1:200 dilution of the epitope-selected rabbit polyclonal antiserum y96. The peptide immunogen for this antiserum, SYMPGPDQSPADSNKTELKC, is located near the COOH terminus of the human AP-2-y protein (11). This peptide was coupled to keyhole limpet hemocyanin before immunization and was also used for the epitope selection and peptide blocking experiments related to the AP-2-y antiserum. Western blot analysis indicated that immunological reagents 3B5 and y96 are specific for AP-2a and AP-2-y, respectively (data not shown). The batch of SC-184 polyclonal antiserum used in these experiments (#E094) reacted equally well with AP-2a and AP-2b and was also capable of recognizing AP-2-y, but with less avidity (data not shown).

After primary antibody incubation, the slides were treated for 30 min with a 1:500 dilution of biotinylated antiserum (10, 11). The peroxidase-substrate complex (Vector Laboratories, Burlingame, CA) was visualized with the chromogenic substrate diaminobenzidine tetrahydrochloride. Subsequently, the slides were counterstained with hematoxylin. Positive AP-2 protein staining was determined by nuclear staining only when it was present within either the BBE or the invasive breast cancer component. The same tumor specimens were also analyzed using a monoclonal antibody to the ERBB-2 protein (Oncogene Science, Uniondale, NY), a polyclonal antibody to the $\beta$ subunit of the IGF-IR (Santa Cruz Biotechnology), and the previously described estrogen receptor immunochemistry assay method for the ER and PR receptors (Abbott Laboratories, Chicago, IL; Refs. 7, 22, and 24). Control samples were processed and stained as described above, except that the primary antibody was omitted, or the primary antibody was preincubated with the appropriate blocking peptide for 1 h before application to the slide.

The pathologist who processed, stained, and graded the specimens was blinded to the clinical histories of the patients. Each slide was rated on the following point scale: (a) 0, no stain; (b) 1+, light stain; (c) 2+, moderate stain; (d) 3+, heavy stain; and (e) 4+, intense staining. The percentage distribution of staining within BBE or the invasive carcinoma component was scored. A value of $\geq 2+$ in intensity was considered positive, and an H-score (defined as the product of intensity and distribution) of $\geq 50$ was used as a cutoff for AP-2a and ERBB-2, and an H-score of $\geq 75$ was used as a cutoff for IGF-IR, ER, and PR expression as described previously (7, 22).

Electrophoretic Mobility Shift Assays. A double-stranded oligonucleotide, 5'GGGGGCGGGGCTCGGGGGGGCCG-3', was synthesized corresponding to nucleotides −97 to −75 in the proximal human IGF-IR promoter (25). The oligonucleotide corresponding to the well-characterized AP-2-binding site in the human metallothionein IIA promoter has been reported previously (19). Both oligonucleotides were labeled with polynucleotide kinase and used as probes for EMSAs or used as cold competitor DNA molecules. EMSA was performed using in vitro-translated AP-2 protein as described previously (19).

Statistical Analysis. All patient data including demographics, age, stage, ER/PR status, lymph node status, histology, radiation therapy technique, and adjuvant systemic chemotherapy as well as outcomes including local, regional, and distant metastasis were entered into a blinded computerized database, and all calculations were performed using the PRODAS database package (Conceptual Software Inc., Houston, TX). The actuarial survival curves were calculated using the life table method, the difference among the curves was determined by the generalized Wilcoxon test, and the differences between the categorical variables were tested by standard $\chi^2$ analysis. The factors affecting survival were determined by a univariate regression analysis according to the Cox proportional hazards regression model. $P \leq 0.05$ was considered to indicate statistical significance.

RESULTS

Established human breast cancer cell lines often express high levels of the AP-2a and AP-2-y proteins (10, 11). We determined whether this phenomenon also occurred in primary breast cancer tissue. For this purpose, we developed specific immunological reagents that were capable of recognizing these two proteins in formalin-fixed paraffin-embedded tissue. We had previously generated mouse monoclonal antibodies 3B5 and 5E4 that are specific for the AP-2a protein (11, 17). We therefore optimized staining procedures so that these two
antibodies could be used for the immunohistochemical analysis of human tissue specimens (see “Materials and Methods”). A representative example of the staining pattern obtained for AP-2α in a human breast cancer biopsy using the 3B5 monoclonal antibody is shown in Fig. 1, A and B. The AP-2α staining pattern was strictly nuclear, which is consistent with the known subcellular localization of this transcription factor. The specific nuclear staining was completely blocked when the antibody was preincubated with its corresponding peptide epitope before immunostaining, demonstrating the specificity of the immune reaction (Fig. 1C). In addition, the nuclear staining pattern was not visualized when breast cancer sections were stained with the secondary antibody alone (Fig. 1D). We also determined that the 5E4 monoclonal antibody, which recognizes a different AP-2α epitope, also generated an equivalent pattern of staining when used on the 5E4 monoclonal antibody, which recognizes a different AP-2α epitope before immunostaining, demonstrating the specificity of these AP-2α immunological reagents.

To study AP-2γ expression, we generated and characterized a novel epitope-selected rabbit polyclonal antiserum, γ96, which does not cross-react with AP-2α or AP-2β (data not shown). The immunoreactivity of breast sections stained with the γ96 antiserum indicated that AP-2γ protein was also localized to the nucleus (Fig. 1E). The staining was not visualized when the tissue sections were stained with the secondary antibody alone (Fig. 1F). Similarly, the specific nuclear staining was blocked when the antibody was preincubated with the relevant peptide before incubation with breast tissue sections (Fig. 1G). Taken together, these studies demonstrate the efficacy of our immunological reagents and indicate that the AP-2α and AP-2γ proteins can be detected in breast cancer biopsies.

**AP-2 Expression in BBE.** The presence of the AP-2α and AP-2γ proteins in a breast cancer specimen might indicate that the expression of these genes had been activated in the progression toward breast cancer. Alternatively, the presence of the AP-2 proteins might indicate that this gene family is normally expressed during the growth and development of BBE. We therefore examined the expression of AP-2α and AP-2γ in 12 BBE specimens from patients undergoing reduction mammoplasty. Both of these proteins could be detected in the ductal epithelium of BBE samples, where they were again predominantly nuclear in localization (data not shown). We found that 3 of 12 (25%) BBE samples stained for AP-2α protein, and 4 of 12 (33%) BBE cases expressed the AP-2γ protein (Table 1). Various combinations of AP-2 protein expression were observed in BBE: (a) AP-2α alone; (b) AP-2γ alone; (c) both AP-2α and AP-2γ; or (d) neither protein detected. We also noted that in comparison to the tumor tissue, both the intensity and distribution of AP-2γ staining were weaker in the BBE than those seen in the invasive cancer specimens (see below).

**AP-2 Expression in Human Breast Cancer.** We next used the specific AP-2 reagents to study the expression of AP-2α and AP-2γ in a well-characterized population of patients for which breast cancer specimens were available. AP-2α protein expression was detected at significant levels in 28 of 80 (35%) breast tumor specimens analyzed (Table 1). Similar levels of AP-2α protein were observed in both the breast cancer specimens and the BBE tissue samples discussed above (P = NS). In contrast, we found that 63 of 75 (84%) breast cancers contained elevated levels of AP-2γ protein, whereas only 33% of BBE cases had AP-2γ immunoreactivity (P = 0.01). This finding indicates that there is a clear up-regulation of AP-2γ expression during the transition from BBE to invasive breast cancer.

We were also able to analyze the data for the coexpression of AP-2α and AP-2γ in 74 of the breast tumor specimens. A total of 65 tumors (88%) contained appreciable levels of at least one of the two AP-2 proteins, and concomitant expression of both proteins was detected in 25 of 74 (34%) tumor specimens (Table 1). Only 9 of 74 (12%) tumors lacked expression of both AP-2α and AP-2γ, which

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**Table 1 AP-2 protein expression in BBE and IDC**

<table>
<thead>
<tr>
<th>BBE</th>
<th>IDC</th>
<th>P</th>
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<tbody>
<tr>
<td>AP-2α+</td>
<td>3/12 (25%)</td>
<td>28/80 (35%)</td>
</tr>
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<td>AP-2α−</td>
<td>9/12 (75%)</td>
<td>52/80 (65%)</td>
</tr>
<tr>
<td>AP-2γ+</td>
<td>4/12 (33%)</td>
<td>63/75 (84%)</td>
</tr>
<tr>
<td>AP-2γ−</td>
<td>8/12 (67%)</td>
<td>12/75 (16%)</td>
</tr>
<tr>
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<td>2/12 (17%)</td>
<td>25/74 (34%)</td>
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<td>37/74 (50%)</td>
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<td>3/74 (4%)</td>
</tr>
<tr>
<td>AP-2α−/AP-2γ−</td>
<td>7/12 (58%)</td>
<td>9/74 (12%)</td>
</tr>
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</table>

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The expression of AP-2α protein in 80 breast cancer tumor specimens and AP-2γ in 75 breast tumors was determined by immunohistochemistry techniques, and nuclear staining was scored independently by a single pathologist using the same histological criteria. An H-score ≥ 50 was used as a cutoff for AP-2 protein expression. Seventy-four samples were analyzed in terms of both AP-2α and AP-2γ expression. Differences in the expression of AP-2 protein between BBE and IDC were determined using x2 analysis.
The high incidence of AP-2α and AP-2γ expression in mammary cancer cell lines (11). Similarly, using an AP-2β-specific antisera, data demonstrate that the majority of breast cancers surveyed also lacked AP-2β expression (data not shown). Taken together, our both AP-2α and AP-2γ, we were able to determine that these samples expression status of AP-2β in the nine tumors that were negative for a lesser extent, AP-2γ. By using this antiserum to examine the materials and Methods) is capable of recognizing AP-2α, AP-2β, and, to but accurate quantification of AP-2β protein is not possible at this time with the available reagents. The SC-184 antiserum (see “Materials and Methods”) is capable of recognizing AP-2α, AP-2β, and, to a lesser extent, AP-2γ. By using this antiserum to examine the expression status of AP-2β in the nine tumors that were negative for both AP-2α and AP-2γ, we were able to determine that these samples also lacked AP-2β expression (data not shown). Taken together, our data demonstrate that the majority of breast cancers surveyed express one or more of the AP-2 proteins, whereas only a small fraction are AP-2 negative. These findings indicate that AP-2 expression may be an important marker for breast tumor progression in vivo. Survival Characteristics of AP-2-expressing Breast Tumors. The high incidence of AP-2α and AP-2γ expression in mammary tumors prompted us to examine whether these genes could act as molecular markers for predicting prognosis. In particular, we were interested in determining whether breast tumors with altered levels of AP-2 were associated with changes in survival rates. The 10-year DDFS rate for breast cancer patients with high levels of AP-2α was 74% compared with 62% for those with low levels of AP-2α (P = NS; Table 2). The 10-year OS for breast cancer patients with high and low levels of AP-2α was 65 and 63%, respectively (P = NS). The 10-year DDFTS for patients with high levels of AP-2γ was 60%, and it was 69% for breast cancer patients with low levels of AP-2γ (P = NS; Table 2). Similarly, there was no difference in the 10-year OS between patients with breast tumors containing high levels of AP-2γ protein and those with breast tumors containing low levels of AP-2γ protein. We also found that the local breast relapse-free survival for breast cancer patients treated with lumpectomy and radiation therapy was not dependent on the level of either AP-2α or AP-2γ, suggesting that AP-2 is not critical in mediating effects related to ionizing radiation (data not shown). Finally, the combined presence of AP-2α and AP-2γ in a tumor sample did not influence the OS or DDFTS.

Hormone Receptor Status and AP-2 Protein Levels. It has been postulated that the AP-2γ protein may be one of the transcription factors controlling ER expression in vitro (12). Therefore, the data obtained for AP-2 expression status were analyzed in terms of the ER and PR positivity of the breast tumors. We found no correlation between AP-2γ expression and ER/PR positivity (Table 3). Similarly, there was no correlation between AP-2α status and PR expression. However, there was a significant correlation between AP-2α status and ER positivity (Table 4). Whereas 15 of 28 (54%) AP-2α-positive tumors were ER positive, only 14 of 52 (27%) tumors that did not express AP-2α were ER positive (P = 0.018). The breast tumors could be further classified into those specimens that coexpressed both AP-2α and AP-2γ (double positives) and those that expressed only one of these two proteins (single positives). ER expression was detected in 13 of 25 (52%) AP-2α/AP-2γ double positive breast cancer specimens and 13 of 40 (33%) AP-2 single positive breast cancer specimens (P = 0.07). In the nine tumors that did not express any AP-2 protein, two were still ER positive, whereas three were PR positive. The latter finding indicated that the presence of AP-2 proteins was not essential for the transcription of these two hormone receptors.

Coexpression of AP-2 and the Oncoprotein ERBB-2. The AP-2 proteins can regulate ERBB-2 promoter activity in vitro, and the expression of ERBB-2 correlates with the presence of one or more AP-2 proteins in human breast cancer cell lines (10, 11). The presence of ERBB-2 protein had previously been detected (7) at elevated levels in 8 of 75 (11%) breast cancer specimens used in the current analysis of AP-2 expression. We found that only 2 of 75 (3%) breast tumors had high levels of ERBB-2 protein when there were low levels of AP-2α (P = 0.015; Table 5). None of the breast tumors had ERBB-2 immunoreactivity when there were low levels of AP-2γ. However, the most striking correlation concerned the expression of ERBB-2 in tumors that contained both AP-2α and AP-2γ protein. Of the 38 tumors that expressed either AP-2α or AP-2γ alone, only 1 had high levels of ERBB-2. In marked contrast, 6 of 24 (25%) AP-2α/AP-2γ

<table>
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<th>Protein expression</th>
<th>10-year DDFTS</th>
<th>10-year OS</th>
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<tr>
<td>AP-2α+</td>
<td>74%</td>
<td>65%</td>
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<td>AP-2α−</td>
<td>62%</td>
<td>63%</td>
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<tr>
<td>AP-2γ+</td>
<td>60%</td>
<td>65%</td>
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<tr>
<td>AP-2γ−</td>
<td>69%</td>
<td>51%</td>
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<th>Protein expression</th>
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<th>10-year OS</th>
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<tr>
<td>ER+</td>
<td>90%</td>
<td>80%</td>
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<tr>
<td>ER−</td>
<td>50%</td>
<td>20%</td>
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<th>Protein expression</th>
<th>ERBB-2+</th>
<th>ERBB-2−</th>
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<tbody>
<tr>
<td>AP-2α+</td>
<td>27/75 (3%)</td>
<td>46/75 (61%)</td>
<td>0.015</td>
</tr>
<tr>
<td>AP-2α−</td>
<td>67/75 (8%)</td>
<td>21/75 (28%)</td>
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</tr>
<tr>
<td>AP-2γ+</td>
<td>0/75 (0%)</td>
<td>11/75 (16%)</td>
<td>0.22</td>
</tr>
<tr>
<td>AP-2γ−</td>
<td>7/70 (10%)</td>
<td>52/70 (74%)</td>
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<td>AP-2α−/AP-2γ−</td>
<td>0/69 (0%)</td>
<td>8/69 (12%)</td>
<td>0.003</td>
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<tr>
<td>AP-2α+ or AP-2γ+</td>
<td>1/69 (1%)</td>
<td>36/69 (52%)</td>
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</tr>
<tr>
<td>AP-2α+ or AP-2γ+</td>
<td>6/69 (9%)</td>
<td>18/69 (26%)</td>
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double-positive tumors expressed ERBB-2 ($P = 0.003$). Our findings strongly support previous in vitro studies that indicate that AP-2 may be an important transcriptional regulator of ERBB-2.

**Coexpression of AP-2 and the IGF-IR.** The correlations found between AP-2, ERBB-2, and ER expression prompted us to examine another signaling molecule that has been implicated in breast cancer, namely, the IGF-IR. Overexpression of the IGF-IR may predict for poor survival characteristics in breast cancer patients, and the receptor has been demonstrated to mediate an antiapoptotic phenotype (26). The presence of the IGF-IR protein had previously been detected (22) in 17 of 75 (23%) breast cancer specimens used in the current analysis of AP-2 expression. Significantly, we found that elevated levels of IGF-IR expression did not occur in the absence of AP-2y ($P = 0.04$; Table 6). Similarly, in the nine samples that were scored as negative for all AP-2 proteins, we did not find sections with high expression of the IGF-IR ($P = 0.08$). In contrast, there was no association between the expression of AP-2a and the levels of the IGF-IR or between AP-2a and AP-2y double positivity and IGF-IR expression. Together, these data indicate that the AP-2 gene family, in particular, AP-2y, may be important for the regulation of IGF-IR expression in human breast cancer.

**Identification of a Proximal AP-2-binding Site in the IGF-IR Promoter.** Previous EMSA studies of the rat IGF-IR promoter indicated that a weak AP-2-binding site might be present between nucleotides −331 and −135 (27). Our further examination of the human and rat IGF-IR promoter sequence revealed that a more proximal AP-2 site might also exist between −90 and −80. This sequence, 5′-GCCCTCGGC-3′, was conserved between both species (25) and matched the previously identified consensus AP-2-binding site, 5′-GCGNNNGGC-3′. When a labeled oligonucleotide corresponding to this IGF-IR sequence was used as a probe for EMSA, a slower-migrating band was produced upon the addition of AP-2a protein. This protein-DNA complex was competed away by an excess of an unlabeled oligonucleotide corresponding to the standard AP-2-binding site from the human metallothionein Ila gene (Fig. 2). Additional competition studies confirmed that the proximal IGF-IR sequence acted as an AP-2-binding site, although it was approximately 10-fold weaker in its ability to bind AP-2 than the standard metallothionein site (data not shown).

**DISCUSSION**

Most breast tumors arise spontaneously in women with no overt family history of breast cancer and do not appear to involve deleterious mutations in BRCA1 and BRCA2 (4). Therefore, it is critical to identify and characterize other genes that may regulate the progression toward mammary carcinoma. One successful approach to identify new genes involved in breast cancer has been to determine how the expression of known prognostic indicators, such as the ER and ERBB-2 genes, are up-regulated in breast cancer cell lines. In vitro studies examining the cis-regulatory sequences responsible for the activation of these two genes have implicated the same transcription factor family: AP-2 (10–12). Our current in vivo examination of AP-2 gene expression in breast cancer specimens also strongly supports a role for these transcription factors in the etiology of human breast cancer. The majority of tumor biopsies examined (88%) express at least one of the AP-2 proteins. This finding largely reflects a significant increase in AP-2y expression in the progression from BBE to IDC. The presence of AP-2a and AP-2y in breast cancer could simply reflect the normal expression of these genes in the ductal epithelia of the healthy mammary gland. Under these circumstances, the AP-2 proteins may represent benign markers of ductal epithelia that play no role in the growth and proliferation of this tissue during the progression to mammary cancer. However, our evidence strongly supports the alternative hypothesis, namely, that the AP-2 genes are intimately involved in controlling growth factor expression in human breast cancer.

We had previously shown that the AP-2 proteins bind to a critical region of the ERBB-2 promoter and are capable of activating ERBB-2 expression in vitro (10, 11). In addition, we had found that ERBB-2 expression in human breast cancer cell lines only occurred in the presence of the AP-2 proteins (10, 11). The data obtained in vivo reinforce the connection between AP-2 and ERBB-2 expression, be-

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**Table 6 Coexpression of AP-2 and IGF-IR**

The expression of AP-2 and IGF-IR in breast cancer lumpectomy specimens was determined by immunohistochemistry techniques. AP-2+ indicates that none of the three AP-2 proteins was detected in a tumor. AP-2+ indicates that at least one of the three AP-2 proteins was present.

<table>
<thead>
<tr>
<th>Protein expression</th>
<th>IGF-IR+</th>
<th>IGF-IR-</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>AP-2α−</td>
<td>14/80 (17%)</td>
<td>38/80 (48%)</td>
<td>NS</td>
</tr>
<tr>
<td>AP-2α+</td>
<td>5/80 (6%)</td>
<td>23/80 (29%)</td>
<td></td>
</tr>
<tr>
<td>AP-2γ−</td>
<td>0/75 (0%)</td>
<td>12/75 (16%)</td>
<td>0.04</td>
</tr>
<tr>
<td>AP-2γ+</td>
<td>17/75 (23%)</td>
<td>46/75 (61%)</td>
<td></td>
</tr>
<tr>
<td>AP−</td>
<td>0/74 (0%)</td>
<td>9/74 (12%)</td>
<td>0.08</td>
</tr>
<tr>
<td>AP+</td>
<td>17/74 (23%)</td>
<td>48/74 (65%)</td>
<td></td>
</tr>
</tbody>
</table>

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**Fig. 2. Identification of an AP-2-binding site in the proximal IGF-IR promoter by EMSA.** The sequences of the IGF-IR and human metallothionein Ila (hMtlla) probes and competitor oligonucleotides are given, and the homology to the AP-2 consensus is shown in bold. All lanes contain the IGF-IR probe and rabbit reticulocyte lysate programmed with AP-2a mRNA. Lane 1, no competitor. Lanes 2–4 contain increasing amounts (1, 10, and 50 ng) of the human metallothionein Ila competitor oligonucleotide.
cause no significant ERBB-2 protein was detected in tumors lacking AP-2. Indeed, the majority of ERBB-2 expression was limited to tumors containing both the AP-2a and AP-2y proteins. Moreover, a reexamination of our previous in vitro data also revealed a high incidence of AP-2α/AP-2γ coexpression in breast cancer cell lines that are ERBB-2 positive (11). One possible explanation for these correlations comes from the finding that the AP-2a and AP-2γ genes can form functional heterodimers in vitro (11). Therefore, the correlation between ERBB-2 expression and AP-2α/AP-2γ double positivity in vivo may indicate that there is a preference for heterodimers between these two transcription factors in the activation of ERBB-2 transcription. It is also noteworthy that ERBB-2 expression was not detected in all mammary tumors that contained both AP-2a and AP-2γ proteins. This finding indicates that the AP-2 transcription factors represent only one component of the regulatory hierarchy required for ERBB-2 promoter activity (28). Although AP-2 expression may not be sufficient for ERBB-2 transcription, our present findings strongly support previous in vitro studies that suggest that the AP-2 proteins are critical transcriptional regulators of ERBB-2 gene expression in breast cancer. A recent study of childhood medulloblastoma also indicated that the AP-2 gene family may be involved in the regulation of epidermal growth factor receptor family gene expression in this class of tumor. In medulloblastomas, the presence of the AP-2 proteins (the various family members were not distinguished) showed significant correlations with the expression of ERBB-2, ERBB-3, and ERBB-4 (29). Therefore, it seems likely that AP-2 transcription factors may play a major role in the regulation of the epidermal growth factor receptor gene family in many different classes of tumor.

Previous studies in breast cancer cell lines had also indicated that AP-2 may regulate ER expression. The AP-2γ protein was isolated from the MCF-7 breast carcinoma cell line based on its ability to interact with an important regulatory element present within the ER gene (12). It has yet to be demonstrated that AP-2 can directly activate ER transcription in vitro, but our in vivo findings support a role for AP-2 in the regulation of ER expression in breast carcinoma. In particular, we detected a significant correlation between AP-2α expression and ER positivity. This finding reveals that AP-2α may be a more important regulator of ER expression than AP-2γ. Finally, with respect to the ER, several tumors were found that expressed this gene in the absence of any AP-2 protein. Therefore, it is apparent that AP-2 is not absolutely required for ER transcription. Nevertheless, the correlations that we observed again link AP-2 with a growth factor signaling pathway that is critical for the outcome of human breast cancer.

Our data also implicate the AP-2 gene family in the regulation of IGF-IR expression in mammary cancer. The IGF-IR is highly expressed in several types of cancer, including breast cancer, and its regulation may be important for the development of malignant transformation (26). The expression of the IGF-IR is hormonally regulated, and the transcription of this gene is also dependent upon transcription factor Sp1 (30). Our data suggest that AP-2 may also be an important regulator of IGF-IR gene expression. In support of this hypothesis, previous studies have shown that AP-2 can interact with the distal region of the IGF-IR promoter (27), and we have now identified a more proximal AP-2-binding site located between nucleotides −80 and −90. Additional studies will be required to determine whether the AP-2 proteins can directly regulate IGF-IR expression, although in this regard, cotransfection experiments have implicated the AP-2α proteins as transcriptional regulators of another insulin-like growth factor regulatory protein, IGFBP-5 (31). Our analysis of breast cancer biopsies indicated that AP-2γ displayed the most significant correlation with IGF-IR gene expression. Taken together with the AP-2α/ER correlation, these data indicate that the closely related AP-2α and AP-2γ transcription factors may have different downstream targets or may differentially regulate the same downstream targets.

The data obtained in our analysis strongly support an important role for the AP-2 proteins in the control of gene expression in breast cancer. The finding that both AP-2α and AP-2γ are expressed to a limited extent in BBE raises the possibility that the AP-2 genes are also required for normal mammary gland development. However, because AP-2α knockout mice die perinatally and lack the ventral body wall, the role of this transcription factor in the normal development and maturation of the mammary gland remains to be determined (16, 17). Similarly, no information is currently available on the role of the AP-2γ gene in mammary gland development. Nevertheless, given the requirement of the AP-2 family for embryonic growth and morphogenesis (16–18), it is striking that the AP-2α and AP-2γ genes are expressed in the ductal epithelia of the normal breast, a tissue that undergoes profound growth and remodeling. We therefore postulate that these genes are involved in normal breast development, and that their function is subverted during the progression toward breast cancer. Further support for the role of AP-2 in mammary gland biology comes from the study of gene expression. In addition to our findings, which demonstrate a connection between AP-2 and the in vivo expression of three growth factor receptors in the breast, important AP-2-binding sites are also associated with the regulatory regions of collagenase, p21waf1/cip1, integrins, cytokeratins, E-cadherin, and mouse mammary tumor virus (32–39). Additional studies are clearly needed to determine how the AP-2 genes are regulated during mammary gland development and to ascertain how these proteins influence the growth and maturation of this organ.

Evidence obtained in vitro concerning the role of AP-2 in cellular transformation suggests two alternative hypotheses for the presence of increased expression of AP-2 in breast cancer. One possibility is that the AP-2 proteins are proliferative signals that activate a variety of growth factor pathways. The inappropriate activation of the AP-2 proteins would thus drive ductal epithelia cells continually through the cell cycle. This theory would be supported by experiments in which overexpression of the AP-2α gene increased the oncogenic potential of PA-1 teratocarcinoma cells (40). An alternative hypothesis is that the AP-2 proteins are more akin to tumor suppressors, and the activation of the AP-2 genes would represent a failed attempt to halt cell proliferation. In this instance, the up-regulation of AP-2 target genes would occur as a secondary consequence of increased AP-2 levels. Several lines of evidence also support this theory. First, transfection of an AP-2α expression construct into HepG2 hepatocarcinoma cells or SW480 adenocarcinoma cells can inhibit their growth and tumorigenicity (38). The observed decrease in cell growth correlated with an increase in the expression of the AP-2 target gene, p21waf1/cip1. Chromosomal mapping of AP-2α also supports a role for this gene as a potential tumor suppressor. The human AP-2α gene, TFA2A, is located at chromosome 6p24, a region associated with loss of heterozygosity in several types of cancer, including follicular center cell lymphoma, ovarian carcinoma, and head and neck squamous cell carcinoma (41–44). It is also noteworthy that studies in cell-free systems have demonstrated that the AP-2α protein can directly interact with SV40 large T antigen, human T-cell lymphotropic virus type I tax, and adenovirus E1A in vitro (36, 37, 45). The ability of these proteins to interact with AP-2 may explain in part how these viral transactivators corrupt normal cellular function. Taken together, these in vitro studies establish a link between the AP-2 proteins, cell cycle control, and cellular transformation. Clearly, additional experiments will be required to understand the in vivo role of the AP-2 gene family in cell fate determination. Knowledge of how the AP-2 proteins...
regulate growth factor signaling in vivo may lead to important applications in the treatment and diagnosis of human breast cancer.

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Expression of AP-2 Transcription Factors in Human Breast Cancer Correlates with the Regulation of Multiple Growth Factor Signalling Pathways

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