Overexpression of TFAP2C in invasive breast cancer correlates with a poorer response to anti-hormone therapy and reduced patient survival

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Abstract

The AP-2gamma transcription factor encoded by the TFAP2C gene is a member of a family of homologous DNA binding proteins that play essential roles during vertebrate embryogenesis but show a restricted pattern of expression in the adult. Elevated expression of the AP-2alpha and AP-2gamma family members has been associated with a number of neoplasms, particularly breast cancer. Here we present an exploratory immunohistochemical study of an archival primary breast tumour series (n = 75) with parallel clinicopathological data using a new, well-characterized antibody to AP-2gamma. Heterogeneous, exclusively nuclear expression of AP-2gamma was found in the epithelial and myoepithelial compartments of normal breast and within tumour epithelial cells. In the breast cancer series, the most notable association was a correlation between elevated levels of AP-2gamma and shortened patient survival (p = 0.0009*). This relationship was also conserved in ER-positive and ErbB2-negative patients; sub-groups generally considered to have a relatively good prognosis. When patient data for survival and duration of treatment response on anti-hormone therapy were examined by multivariate analysis, AP-2gamma was revealed in this study to be an independent predictor of outcome for both survival (p = 0.005) and response to anti-hormone therapy (p = 0.046). Studies using in vitro models confirmed that while tamoxifen response is associated with lower levels of AP-2gamma, acquisition of resistance to this and other anti-hormone measures (eg faslodex or oestrogen deprivation) is associated with high levels of nuclear AP-2gamma. Together these data suggest that elevated tumour AP-2gamma expression can contribute to the failure of cells to growth arrest following anti-hormone treatment and lead to sustained growth and poorer patient outcome.
Overexpression of *TFAP2C* in invasive breast cancer correlates with a poorer response to anti-hormone therapy and reduced patient survival

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**Running title:** Poor survival and response in AP-2γ overexpressing breast tumours
ABSTRACT

The AP-2γ transcription factor encoded by the TFAP2C gene is a member of a family of homologous DNA binding proteins that play essential roles during vertebrate embryogenesis but show a restricted pattern of expression in the adult. Elevated expression of the AP-2α and AP-2γ family members has been associated with a number of neoplasms, particularly breast cancer. Here we present an exploratory immunohistochemical study of an archival primary breast tumour series (n=75) with parallel clinicopathological data using a new, well-characterised antibody to AP-2γ. Heterogeneous, exclusively nuclear expression of AP-2γ was found in the epithelial and myoepithelial compartment of normal breast and within tumour epithelial cells. In the breast cancer series, the most notable association was a correlation between elevated levels of AP-2γ and shortened patient survival (p=0.0009*). This relationship was also conserved in ER positive and ErbB2 negative patients; sub-groups generally considered to have a relatively good prognosis. When patient data for survival and duration of treatment response on anti-hormone therapy were examined by multivariate analysis, AP-2γ was revealed in this study to be an independent predictor of outcome for both survival (p=0.005) and response to anti-hormone therapy (p=0.046). Studies using in vitro models confirmed that while tamoxifen response is associated with lower levels of AP-2γ, acquisition of resistance to this and other anti-hormone measures (e.g. faslodex or oestrogen deprivation) is associated with high levels of nuclear AP-2γ. Together these data suggest that elevated tumour AP-2γ expression can contribute to the failure of cells to growth arrest following anti-hormone treatment and lead to sustained growth and poorer patient outcome.

Keywords: AP-2 transcription factors, breast cancer, tamoxifen resistance, prognostic markers, immunocytochemistry
INTRODUCTION

The AP-2 family of developmentally regulated transcription factors consists of five members, AP-2α-ε, [1-4] that are expressed during vertebrate embryogenesis in overlapping but distinct patterns of expression, especially within tissues undergoing morphogenesis [1]. The importance of these expression patterns has been borne out by homologous gene inactivation studies in mice. Mice lacking AP-2α showed multiple defects in the development of neural crest-derived tissues such as the cranio-facial skeleton [5-7] and the outflow tract of the heart [8]. Loss of AP-2β led to failed terminal kidney differentiation [9]. In contrast, AP-2γ was shown to be absolutely required within the extra-embryonic lineages for early post-implantation development [10, 11]. The more recently described AP-2δ and AP-2ε genes are thought to play more specialised roles in embryo brain and CNS [3, 12].

Expression of AP-2 proteins in normal adult tissues is much more restricted. However, a large number of studies have clearly demonstrated expression of AP-2α and AP-2γ, in particular, in a variety of solid tumours, including breast cancer where transcriptional targets include ERBB2, oestrogen receptor (ERα), and p21WAF1/CIP1 (reviewed [13]). Expression of AP-2α in breast cancer has generally been associated with a favourable outcome. For example, we and others have found a direct correlation between AP-2α and expression of ERα and the cell cycle inhibitor p21WAF1/CIP1, coupled with a lower rate of proliferation [14, 15]. AP-2α staining was found to be highest in normal breast epithelia with a decline in levels in samples of DCIS and the lowest expression in cases of invasive cancer [14]. Reduced nuclear AP-2α staining has also been associated with more aggressive, high-grade breast tumours [16] and TFAP2A mRNA expression has
been inversely correlated with tumour grade [17]. In summary, AP-2α expression in tumours has been associated with favourable prognostic markers which has led to the suggestion that this factor may be acting in a tumour suppressive role, in breast cancer and in other cancer types (reviewed [13]).

In contrast, elevated tumour levels of AP-2γ have largely been correlated with an adverse phenotype and increased disease progression. High levels of AP-2γ were found in testicular cancer [18], germ cell tumours [18, 19], advanced stages of ovarian cancer [20] and certain stages of melanoma [21]. In breast cancer, data mining of microarray expression studies has shown an association between high levels of TFAP2C mRNA and higher clinical grade and shorter survival times [17, 22]. However, as reviewed recently [13], the few studies that examined protein levels in breast cancer rarely used specific immunological reagents against AP-2γ, leading to apparently conflicting observations and a lack of clarity as to its overall role. In the current exploratory study, therefore, we have developed and carefully characterised a monoclonal antibody against AP-2γ and used it to examine expression levels in a series of primary invasive breast tumour samples (previously assayed for AP-2α [14]) to determine if AP-2γ correlates with outcome. We have then further explored the relationship between AP-2γ endocrine response using breast cancer models.

METHODS

Cell lines

Acquired tamoxifen-resistant derivatives of MCF-7 (TamR) and T47D (T47D-R), MCF-7 cells with acquired resistance to faslodex (FASR-LT) or to severe oestrogen and growth factor deprivation (MCF-7X) [23-26] were examined in vitro.
Hormonal manipulation was carried out by supplementing standard growth medium (phenol-red-free RPMI medium containing 5% charcoal-stripped foetal calf serum, 200mM glutamine, 10 IU/ml penicillin, 10μg/ml streptomycin) with either $10^{-8}$ β-oestradiol, $10^{-6}$M 4-hydroxytamoxifen or $10^{-7}$M faslodex for 48 hours prior to cell harvest. Log-phase MCF-7, T47D, ZR75-1, MDAMB453 and MDAMB436 were also prepared for assay optimisation. For immunocytochemistry, cells were concentrated by centrifugation, transferred to 2% low melting point agarose/PBS at 37°C to form solid pellets, fixed in 4% formal saline and embedded in paraffin wax. Pellets were cored and assembled in replicates using a tissue arrayer forming a composite array which was sectioned at 5μM for immunostaining.

**Generation of AP-2γ monoclonal antibody, 6E4**

A 30 kDa AP-2α/γ fusion protein (see Figure 1A) was expressed in bacteria and purified [27]. Balb/c mice received four subcutaneous injections of purified protein (25μg/injection) with adjuvant at four weekly intervals.

**Electromobility shift assays (EMSA) and Western blotting**

AP-2 proteins, synthesized in vitro (T’N’T kit; Promega) from wild-type and mutant AP-2 clones [28], were used in EMSA assays as previously described [29]. IVT proteins or whole cell lysates (10μg/ lane) were blotted to Hybond-ECL membrane (Amersham) and probed with 6E4. Chemiluminescent detection of horseradish peroxidase-conjugated secondary antibodies was performed using the West Femto Maximum Sensitivity Substrate kit (Pierce). Loading was controlled by
blotting for nuclear antigens Ku-70 (C-19, Santa Cruz) or PCNA (P10, Santa
Cruz).

**Clinical samples**

A series of 75 formalin-fixed, paraffin-embedded primary tumours obtained from patients (age range 25-77, median 53.5) with histologically proven breast cancer presenting for surgery at Nottingham City Hospital between 1984-1987 was investigated. This is a well-characterised, historical breast cancer series with approval for use without further patient consent (approved ethics application, Nottingham Research Ethics Committee 2; C108030; [30]). All patients received systemic antihormonal therapy for locally-advanced primary carcinoma (>5cm) or metastatic disease, or for recurrence after surgery alone. Premenopausal patients (n=23) received Zoladex® (3.6mg depot/28days) alone or with tamoxifen (20mg twice daily), while postmenopausal (n= 52) received only tamoxifen. None of the patients had received any form of adjuvant cytotoxic therapy or prior endocrine treatment. All patients had lesions assessable for response quality at 6 months according to UICC criteria (responders (CR, PR, SD) n=28; progressors n=47). Survival and duration of anti-hormone response were measured from commencement of anti-hormone to death or progression on therapy respectively. Median follow-up was 16 months (see supplementary information for further details). Additional samples assayed were: i) pure DCIS (n=20; comedo or non-
comedo) of known ErbB2 status and ii) normal breast reduction mammoplasty material (n=20).
Optimised Immunohistochemistry assay, scoring and analysis

All sections were de-waxed, re-hydrated and endogenous peroxidases were blocked using 3% hydrogen peroxide (5 min). Sections were microwaved (0.01M citrate buffer, pH 6 for 30 min at 560W) to aid antigen retrieval. All subsequent steps were at 25°C. A 1/35 dilution of 6E4 conditioned medium was applied overnight and, after washing, secondary antibody (biotinylated anti-mouse immunoglobulins in 1% BSA/PBS; Biogenex Super Sensitive “link”) was applied (1/40; 60 min). Tertiary reagent (streptavidin peroxidase in 1% BSA/PBS; Biogenex Super Sensitive “label”) was applied (1/40; 60 min) followed by diaminobenzidine/hydrogen peroxide chromogen for 10 min with methyl green (0.5%) counterstaining. Negative controls were incubated with mouse isotype-specific control immunoglobulins(Dako).

All sections were assessed simultaneously by two observers blinded to the clinical data using a dual-viewing light microscope with x40 lens. Areas of tumour-associated-normal/-DCIS were excluded from the assessment of invasive tumour immunostaining. Matched control slides were checked for non-specific binding before assessing the nuclear staining intensity and percentage positivity of the tumour epithelial cells (minimum of 2000 tumour cells evaluated). The data were used to construct an AP-2γ histopathology score (HScore; range 1-300) for each tumour specimen as detailed previously [31], with epithelial staining also examined in DCIS and normal breast sections.

Mann-Whitney U-test was used to compare AP-2γ levels in comedo versus non-comedo DCIS and to examine tumour AP-2γ staining in relation to ER or ErbB2 status, response quality, menopausal status, disease site (locally-advanced vs.
metastatic disease), tumour grade (1/2 vs. 3), PR and AP-2α/β status [14].

Univariate analysis was performed using the Kaplan-Meier method with a log rank test. Multivariate analysis was performed using Cox's Proportional Hazards model, controlling for co-variates as listed in the Results. Univariate and multivariate analysis addressed AP-2γ impact on survival from initiation of therapy or on duration of therapeutic response, before and after subdivision for (i) ER or (ii) ErbB2 status. p<0.05 was considered significant (*). No adjustments for multiple testing were made due to the exploratory nature of the study.

Results

Characterisation of AP-2γ monoclonal antibody

A bacterially expressed AP-2α/γ fusion protein (Figure 1A) was used to generate an immunogen for injection into mice. One mouse with a weak response against AP-2α but a strong response against AP-2γ was selected for monoclonal antibody generation. Of the hybridoma lines subsequently derived, 6E4 interacted strongly and specifically with AP-2γ with no cross reaction to other AP-2 family members (Figure 1B). The epitope was mapped within the C-terminal 20 amino acids of AP-2γ, as shown by "supershifting" EMSA: migration of protein/DNA complexes with AP-2γ mutant ∆N220C430 was not altered by addition of 6E4 (Figure 1C, compare odd and even lanes). 6E4 does not detect murine AP-2γ (data not shown) which differs by two amino acids within this C-terminal region. Western blotting (Figure 1D) of lysates from breast tumour-derived lines of known AP-2γ expression status [32] showed a clear 50 kDa band in expressing lines (MCF-7, T47D, ZR75-1) which was absent in non-expressing cells (MDAMB453 and
MDAMB436). There were essentially no other cross-reacting species, indicating that 6E4 is a highly specific reagent suitable for evaluation of AP-2γ immunochemistry.

**AP-2γ Immunocytochemistry**

Paraffin sections of cell pellets from breast tumour-derived lines were used to initially optimise AP-2γ staining. Intense nuclear staining was only present in AP-2γ positive lines (MCF-7, HScore = 180; T47D, HScore = 130; ZR75-1, HScore = 110). Lines negative for AP-2γ exhibited virtually no nuclear staining (MDAMB453; HScore = 35 and MDAMB436; HScore = 15). This staining profile closely reflected the relative band intensity observed by Western blotting (Figure 1D) thus validating the immunostaining methodology.

A cohort of 75 primary breast tumours which had previously been examined for expression of a number of other markers, including AP-2α/β [14], was assayed for AP-2γ expression. A continuum of nuclear immunostaining levels between the samples was observed (HScore range 20-230) with no obvious cut-off point in staining distribution (see supplementary figure). The median (HScore ≥ 120) was used to define those tumours with substantial AP-2γ “positivity” versus “negative” (low staining) samples for univariate analyses, but absolute HScore values were used for all other analyses.

Tumour epithelial staining was heterogeneous and entirely confined to the nuclei: no cytoplasmic immunostaining was apparent and surrounding stromal cells and blood vessels were negative (see Figure 2A). A proportion of the samples
contained adjacent regions of tumour-associated DCIS (TAD; n = 19; see Figure 2B) and/or tumour-associated normal epithelium (TAN; n = 32) which were also positive for AP-2γ expression. To examine these staining patterns further, we stained twenty cases of pure DCIS (10 comedo, 10 non-comedo) and saw higher AP-2γ staining (p = 0.015*) in the comedo (Median HScore = 95, range = 40-170; Figure 2C) compared to the non-comedo samples (Median HScore = 23.5, range = 2-125; Figure 2D). Notably, the former group were enriched for ErbB2 (50% overexpressors) relative to their non-comedo counterparts (no overexpressors). Twenty samples of normal breast tissue from reduction mammoplasty patients were also examined. Staining was again nuclear and heterogeneous with apparently a higher proportion of intensely staining cells than seen in invasive tumours or DCIS (Figure 3); this was particularly prominent and more homogeneous in the myoepithelium (see inset, Figure 3).

**Elevated AP-2γ expression is associated with reduced patient survival**

Examination of AP-2γ expression levels in this tumour series revealed a correlation between elevated AP-2γ and shortened survival from initiation of therapy (Table I). We found a median survival of 39 months for cases with low AP-2γ as compared to 15 months for those with high AP-2γ expression (p=0.0009*). This is illustrated in Figure 4, where a marked separation in survival curves is evident after 20 months. Clinically, patients are now stratified according to ER and ErbB2 status; we thus examined AP-2γ in the ER positive and the ErbB2 negative patient sub-groups, noting a significant relationship between higher expression and shortened survival in both (Table I). These relationships in
this patient series are intriguing given that ER positivity and ERBB2 negativity are generally considered as favourable prognostic indicators in breast cancer and suggest that nuclear AP-2γ positivity may be a marker of poorer outlook in this heterogeneous disease.

We also observed increased AP-2γ associated with shortened time to progression (TTP; 3 versus 9 months for AP-2γ “negative”; p=0.04*). Ag PubMed ain, this relationship was significant in ErbB2 negative patients (3 versus 10 months for AP-2γ high and low expressors respectively; p=0.002*), where examination of response quality at 6 months also revealed responders had lower AP-2γ than progressors (median HScore=95 and 135 respectively; p=0.013*).

Survival and TTP on anti-hormone therapy were additionally examined by multivariate analysis using Cox proportional hazards model. In addition to AP-2γ, co-variates considered for inclusion were ER, PR and ErbB2 status, site, grade and menopausal status. Univariate analysis revealed parameters significantly associated with survival in the series were grade (p=0.003), site (p<0.001) and ER status (p<0.001). These parameters were also significant in relation to TTP (p<0.001, p<0.001 and p<0.001 respectively) but additional factors were menopausal (p=0.005) and PR status (p=0.017). ErbB2 was not significant in either analysis. Significant co-variates were subsequently used to build survival and TTP models in multivariate analysis (Table II). AP-2γ was an independent predictor of poorer survival, (p=0.005*) and also of decreased duration of response on anti-hormone therapy (p=0.046*). The association between increased hazard ratio and AP-2γ was retained in ErbB2 negative disease for
survival (p=0.002*) and duration of response (p=0.002*). In ER positive patients, AP-2γ again related to reduced survival (p=0.048*), although associations with TTP were lost in this small series.

**AP-2γ expression in in vitro models is associated with anti-hormone resistance**

TFAP2C has been shown to be a modestly oestrogen-responsive gene [33]. To explore the relationship further between AP-2γ levels and response versus resistance to anti-hormone therapy, we used a panel of well-characterised in vitro breast cancer models established in ER positive, ErbB2 negative (by IHC criteria) cell lines. Western blotting demonstrated that AP-2γ levels declined in MCF-7 cells treated with tamoxifen for 48 hours compared to cells maintained in oestrogen-containing media (Figure 5, compare lanes 2 and 1). Interestingly, the ER-positive, tamoxifen resistant (TamR) derivative of these cells showed fully-restored levels of AP-2γ which were maintained even when these cells were treated with the pure anti-oestrogen, faslodex (Figure 5, compare lanes 3 and 4).

Localisation of AP-2γ in in vitro models of anti-hormone resistance was also investigated by immunostaining paraffin-embedded pellet arrays. As shown in Figure 6, hormonally manipulated MCF-7 and also T47D cells expressed varying levels of AP-2γ but in all cases the protein remained predominantly nuclear. While 17β-estradiol was stimulatory, acute addition of tamoxifen again reduced AP-2γ staining in MCF-7 cells (Figure 6, compare panels A, B and C). However, cells which had become refractory to tamoxifen showed fully-restored wild-type levels of nuclear AP-2γ that were again maintained when these cells were exposed to faslodex (Figure 6, compare Panels D, E with A). T47D cells extensively cultured in tamoxifen also
expressed wild-type levels of AP-2γ (Figure 6, panels H and I). Moreover, the MCF-7X model (resistant to severe oestrogen deprivation) and acquired faslodex resistant cells also displayed readily-detectable levels of nuclear AP-2γ, with particularly substantial levels in the former (Figure 6, compare panels F, G and A).

Thus, while AP-2γ levels clearly fall during tamoxifen response, these experimental models demonstrate that breast cancer cells re-instigate substantial levels of nuclear (and therefore potentially active) AP-2γ when they become anti-hormone resistant. Together with our observational study associating higher AP-2γ levels with poorer outcome in anti-hormone treated patients, these data suggest a potential contribution for AP-2γ in the biology of diverse, anti-hormone-resistant states.

DISCUSSION

Studies examining expression of the AP-2γ transcription factor in tumours have often used reagents that are either poorly characterised or which recognise multiple AP-2 family members [13]. Here we describe the generation and extensive characterisation of a specific monoclonal antibody against AP-2γ and its application to examine factor expression levels in normal and malignant breast tissue. Strong AP-2γ staining was noted in the nuclei of both epithelial and myoepithelial cells of the pure normal breast samples, with a marked mosaic expression in normal luminal epithelial cells (Figure 3), which reflects findings in the terminal end buds (TEBs) of the developing mouse mammary gland. TEBs ultimately form the ductal network and contain two cell populations: body cells which will form ductal epithelial tissue and cap cells which are the progenitors of the myoepithelial layer. A mosaic, rather than uniform, pattern of expression for
AP-2γ was noted in both cell types in mouse TEBs [34].

Compared to the level of staining in normal tissue, we see an apparent reduction in AP-2γ nuclear staining between normal epithelial cells and either DCIS or invasive tissue. Similar data was noted from microarray studies where AP-2γ was shown to be expressed at a significantly higher level in samples of normal tissue or benign disease than in cases of ductal carcinoma ([22, 35]; p = 0.002 and 0.009 respectively). Our most striking finding however, is that, for those patients whose tumours retained high levels of nuclear AP-2γ expression, there appears to be an association with reduced patient survival from initiation of therapy in this series (Figure 4). This relationship is retained in ER positive and ErbB2 negative cases, two patient sub-groupings generally considered to have a relatively good prognosis in breast cancer. Since high levels of AP-2γ occur in normal breast epithelial cells, elevated expression of this factor per se is unlikely to be a tumour initiating event. However, our data imply a positiverole for this factor in facilitating adverse behaviour of cancers that arise in AP-2γ expressing epithelial cells. Thus, AP-2γ may prove to be a marker for patients with an otherwise favourable prognosis who fail to respond to therapy. In support of this, univariate and multivariate analyses indicated that AP-2γ status was an independent predictor of poor outcome, particularly for survival, but also for progression on anti-hormone therapy in this series.

Few previous reports have specifically examined AP-2γ expression in breast cancer but, in agreement with this study, a qPCR analysis of 38 primary breast tumours found a significant association between elevated TFAP2C mRNA and reduced
disease-free survival, although an association with overall survival was not observed [36]. Two other studies using different polyclonal AP-2γ antibodies failed to find a link with outcome. One study focused on patients with early-stage disease treated with lumpectomy [15] and in the second study the commercial antibody employed apparently only stained the myoepithelial compartment [37]. In contrast, using gene expression profiling, TFAP2C mRNA expression was associated with advancing clinical grade (p<0.002, [17]). It was also found to be significantly higher in patients who died within 5 years of breast cancer compared to those patients who were still disease free after 5 years ([22]; p=0.0177), while AP-2α mRNA expression levels were not significantly different between these two patient groups.

Our study further highlights the opposing roles seemingly played by the two AP-2 family members in breast cancer. AP-2 factors can regulate expression of the cell cycle inhibitor p21^{WAF1/CIP1} and ERα [1, 13] and for AP-2α this relationship may underlie associations between elevated levels of this factor and an indolent breast cancer phenotype. In contrast, elevated AP-2γ expression has been shown here to be an independent predictor of poorer survival, where its expression did not relate to AP-2α/β status as previously determined for this series [14]. Interestingly, although mammary-directed overexpression of AP-2γ in transgenic mice did not lead to tumour development, marked epithelial hyperplasia was observed [38] implicating AP-2γ in the promotion of breast epithelial cell proliferation.

High levels of nuclear AP-2γ may also be associated with an anti-hormone resistant phenotype, as suggested by our clinical and in vitro findings and also supported by a recent study that examined AP-2γ levels within a series of ER+ patients with known
response to tamoxifen. Multivariate analysis revealed AP-2γ to be an independent marker of resistance to tamoxifen, particularly in postmenopausal patients [39]. Failure of, or resistance to, anti-hormone therapy remains a major problem in the clinical management of breast cancer and several mechanisms have been proposed to account for this resistance that can equate with poor prognosis [40]. As exemplified by our TamR models [23, 41], essentially all of these mechanisms lead to the activation of alternative signalling pathways that bypass cell cycle arrest and allow cells to continue to proliferate and progress to more aggressive states. In ER+ patients, AP-2γ as a ligand-activated ERα target gene [33] would be expected to be down-regulated by tamoxifen. However, poor anti-hormone response in tumours with high nuclear AP-2γ might be associated with failed ERα regulation of TFAP2C transcription. In support of this, we have shown (Figures 5 and 6) that ER+, TamR cells no longer down-regulate AP-2γ expression in the presence of tamoxifen and instead express high levels of nuclear, potentially active protein. Restored nuclear levels of AP-2γ were also observed in lines resistant to faslodex or severe oestrogen deprivation, suggesting that the prevailing AP-2γ level in all these models maintains a gene expression profile that contributes to the failure of the cells to growth-arrest following anti-oestrogen treatment. By extension, breast tumours that overexpress AP-2γ may similarly sustain growth during therapy contributing to the poor patient outcome observed in the tumour series.

Since only 75 patients were examined in this study, it must be deemed exploratory, where future confirmatory studies in larger clinical breast cancer sets with associated endocrine response and survival data will be imperative. Nevertheless, the findings of the present study are clearly important since they suggest that AP-2γ
expression may be an indicator of reduced patient survival in breast cancer and that this factor therefore plays an opposite role to that of AP-2α in this disease. AP-2γ expression may also equate with a lack of response to anti-hormonal therapy, as supported by our in vitro findings.

Acknowledgements
We are indebted to the CR-UK research monoclonal antibody service and the late Jane Steel for help in generating 6E4; to Dr. Matthew Grainge (Epidemiology & Public Health, Queen's Medical Centre, Nottingham) for expert statistical advice; and to Pauline Finlay and Lynne Farrow for technical and statistical assistance. We are grateful to Prof. Louise Jones for comments on the manuscript and we acknowledge generous funding from Cancer Research UK and the Tenovus charity.

Abbreviations: EMSA: electromobility shift assay; ER: estrogen receptor; IVT: in vitro translated (protein); aa, amino acid(s); SERMs: selective estrogen receptor modulators; wt: wild-type; DCIS: ductal carcinoma in situ
### TABLES

#### Table I: Median survival

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<th>Patient Group</th>
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<th>Median survival (months)</th>
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<td>15</td>
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Univariate analysis examining the relationship between AP-2γ expression level (relative to the median HScore) and survival from initiation of therapy in three patient groups. CI = confidence interval.
<table>
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<th>Predictor</th>
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<td>(n=55, events=45)</td>
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<tr>
<td>AP-2γ</td>
<td>1.7 [1.22 - 2.39]</td>
<td>0.002</td>
</tr>
<tr>
<td>ER status</td>
<td>2.3 [1.14 - 4.63]</td>
<td>0.02</td>
</tr>
<tr>
<td>Site</td>
<td>2.66 [1.35 - 5.21]</td>
<td>0.005</td>
</tr>
<tr>
<td>Grade</td>
<td>-</td>
<td>ns</td>
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<tr>
<td><strong>Time to Progression</strong></td>
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</tr>
<tr>
<td><strong>AllPatients</strong></td>
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<td>(n=73, events=61)</td>
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<td>&lt;0.001</td>
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<tr>
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<td>&lt;0.001</td>
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<tr>
<td>Grade</td>
<td>2.11 [1.18 - 3.7]</td>
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<tr>
<td>PR status</td>
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<td>ns</td>
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<tr>
<td>Menopausal status</td>
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<td><strong>ErbB2 -ve Patients</strong></td>
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<td>(n=54, events=45)</td>
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<tr>
<td>AP-2γ</td>
<td>1.77 [1.24 - 2.54]</td>
<td>0.002</td>
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<tr>
<td>ER status</td>
<td>-</td>
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<tr>
<td>Site</td>
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<td>Menopausal status</td>
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Multivariate analysis using absolute HScore values for AP-2γ expression in relation to survival from initiation of therapy to time to progression. Hazard ratio and CIs for AP-2γ should be interpreted as the increase in risk associated with an increase of 1 SD in the co-variate (mean = 113; SD = 45.5; ns = not significant). Cut points for positive status were: ER HScore ≤0.05, PR Hscore ≤0.02, ErbB2 overexpression of membrane staining.
FIGURE LEGENDS

Figure 1: Specificity of 6E4 for AP-2γ. A) Fusion protein antigen consisting of the truncated N-terminus of human AP-2α (aa 165 to 223) fused to the C-terminal sequence of human AP-2γ (aa 278-440). An initiator Met was engineered at the start (in bold); the sequences switch within the AP-2 basic domain at a common Leu residue (underlined). B) Wild-type (wt) AP-2α, β and γ factors, plus the ΔN220 mutant of AP-2γ, were synthesised in vitro (IVT), blotted to membrane and probed with hybridoma 6E4. C) EMSA assay of IVT protein from AP-2γ wt (lanes1-2), ΔN220 (lanes 3-4) or ΔN220C430 (lanes 5-6) bound to a consensus AP-2 binding site probe in the presence (lanes 2, 4, 6) or absence (lanes 1, 3, 5) of 6E4. D) Whole cell extracts (10μg/lane) from AP-2γ positive (lanes 2-4) and negative (lanes 5-7) cell lines (all breast tumour-derived except HepG2, a known AP-2 negative liver carcinoma line) were separated by SDS-PAGE and blotted to membrane. Wt AP-2γ IVT protein was used as a size marker (lane 1). The blot was probed sequentially with 6E4 and for Ku-70 (loading control). Migration of size markers is indicated on the left.

Figure 2: Immunohistochemical staining patterns for AP-2γ in breast tumours. Examples of staining with antibody 6E4 in formalin-fixed, paraffin-embedded breast tumours (panels A and B) and pure DCIS (panels C and D); all original magnification x20. A) Invasive ductal carcinoma with positive nuclear staining for AP-2γ surrounded by negative stroma; B) AP-2γ positive invasive tumour and associated DCIS (arrowed) with negative stroma. Representative staining in comedo (C) and non-comedo (D) DCIS.

Figure 3: Immunohistochemical staining patterns for AP-2γ in normal breast. Staining of epithelial and myoepithelial cells in normal ducts from a reduction mammoplasty sample (original magnification x20). Inset = detail from a duct showing intense staining in the myoepithelial layer (M) and more heterogeneous staining of the luminal (L) layer.

Figure 4: Correlation between AP-2γ expression and poorer prognosis in invasive breast tumours. Kaplan-Meier survival curve for AP-2γ expression
≥ (High $\gamma$) versus < (Low $\gamma$) the median HScore of 120. The two-sided p value was calculated using log rank testing.

**Figure 5: Expression of AP-2$\gamma$ in wild-type and tamoxifen-resistant MCF-7 cells.** Western blot of whole cell lysates (10$\mu$g/lane) from wild-type MCF-7 cells grown in oestrogen-containing media (lane 1) or with 4-OH tamoxifen treatment for 48 hours (lane 2) compared with acquired tamoxifen resistant (TamR) cells maintained in 4-OH tamoxifen-containing medium alone (lane 3) or supplemented with Faslodex for 48 hours (lane 4). The blot was probed sequentially with 6E4 and for PCNA (loading control) as indicated.

**Figure 6: AP-2$\gamma$ remains nuclear in hormonally manipulated breast cancer cells and their anti-hormone resistant variants.** Immunohistochemical staining for AP-2$\gamma$ in wild-type and acutely or chronically hormone manipulated MCF-7 and T47D cells. Wt MCF-7 cells in 5% stripped serum experimental medium (A) or supplemented with β-oestradiol (B) or 4-OH tamoxifen (C). Acquired tamoxifen resistant MCF-7 cells (TamR) in the presence of 4-OH tamoxifen (D) or with faslodex (E). MCF-7 cells with acquired resistance to faslodex (F) or MCF-7X cells resistant to severe oestrogen and growth factor deprivation (G). Wt (H) and acquired tamoxifen-resistant (I) T47D cells. Original magnification x40.

**Supplementary Figure: Distribution curve of AP-2$\gamma$ immunostaining in the breast tumour series.** The graph records the AP-2$\gamma$ HScore (range 20 – 230) assigned to 75 primary breast tumours. Median HScore = 120.
References


Additional information about the patient series (for on-line publication)

The study series is a historic, mixed patient group comprising:

(i) patients presenting with primary disease that was either locally-advanced (i.e. with large tumours >5cm) or metastatic who were treated with surgery immediately followed by endocrine therapy.

(ii) patients presenting with primary disease that, following surgical treatment alone, recurred with metastatic disease. These patients were then treated with endocrine therapy for this metastatic disease.

These samples have been used in many immunohistochemical studies to explore if there is a relationship between the clinical impact of first exposure to endocrine therapy (including survival measured from initiation of treatment) and the biomarker under test (in this instance AP-2γ) as measured in the primary tumour.

At the time of accumulation of this series (1984-1987), the prevailing view was that adjuvant endocrine therapy did not affect survival [1] and this did not become routine practice until the Oxford overview [2], explaining why this study also includes patients initially treated with surgery only for their primary disease. During this period patients with ER negative disease were also considered for a trial period on endocrine therapy, as up to 10% response rates were occasionally observed. Thus, all patients in the study were treated with tamoxifen-based regimes, as determined by menopausal status (see article text). Since 90% of the ER- patients did not respond to endocrine therapy they may have been considered for chemotherapy once they progressed. However, there is no detailed log available for the chemotherapeutic regimens that might have been subsequently employed or indeed which, if any, ER- patients received such treatment. Moreover, since ErbB2 was not routinely measured at the time (this was examined later for this series as an exploratory marker), potential use of chemotherapy cannot be extrapolated from this status.

Further data were available for this series including patient age, menopausal status, disease site (locally advanced versus metastatic disease), tumour type and grade (nuclear pleomorphism, tubular differentiation, mitotic activity), PR and ER status (ER+ n=45; ER- n=30), as well as proliferative capacity (Ki67) and
immunohistochemical expression of ErbB2 plus AP-2α/β staining from previous study of this series (see [3, 4] and references therein). Information about the number of metastatic sites and tumour size was not available. Variables not found to be significant from initial univariate analysis were excluded from further investigation. Notably, we have compared the data on AP-2α and AP-2γ expression levels in this patient series: 63 samples had an HScore available for both markers but there was no significant relationship in the All, ER+/- nor ErbB2+-/- patient groups. Thus, the association we report here between AP-2γ and survival appears (given the patient set size) to be entirely independent of AP-2α expression level.

Figure 1
Figure 4

Survival in All Patients (n=75)

Low $\gamma$

High $\gamma$

$p = 0.0009$

Survival (months)

Survival (months)
Figure 5
Supplementary Figure