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Expression of phosphorylated eIF4E-binding protein 1, but not of eIF4E itself, predicts survival in male breast cancer

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Background: Male breast cancer is rare and treatment is based on data from females. High expression/activity of eukaryotic initiation factor 4E (eIF4E) denotes a poor prognosis in female breast cancer, and the eIF4E pathway has been targeted therapeutically. Eukaryotic initiation factor 4E activity in female breast cancer is deregulated by eIF4E overexpression and by phosphorylation of its binding protein, 4E-BP1, which relieves inhibitory association between eIF4E and 4E-BP1. The relevance of the eIF4E pathway in male breast cancer is unknown.

Methods: We have assessed expression levels of eIF4E, 4E-BP1, 4E-BP2 and phosphorylated 4E-BP1 (p4E-BP1) using immunohistochemistry in a large cohort of male breast cancers ($n = 337$) and have examined correlations with prognostic factors and survival.

Results: Neither eIF4E expression nor estimated eIF4E activity were associated with prognosis. However, a highly significant correlation was found between p4E-BP1 expression and disease-free survival (DFS), linking any detectable p4E-BP1 with poor survival (univariate log rank $P = 0.001$; multivariate HR 8.8, $P = 0.0001$).

Conclusions: Our data provide no support for direct therapeutic targeting of eIF4E in male breast cancer, unlike in females. However, as p4E-BP1 gives powerful prognostic insights that are unrelated to eIF4E function, p4E-BP1 may identify male breast cancers potentially suitable for therapies directed at the upstream kinase, mTOR.

Male breast cancer (MBC) is a rare disease, accounting for less than 1% of all breast cancers and less than 1% of all male cancers diagnosed in the UK in 2009 (CRUK, 2010). There is relatively little research into MBC, presumably as a result of its rarity, and much of the published work has focused on comparisons with female breast cancer (FBC). Comparisons demonstrate that MBC is more likely to be estrogen receptor positive (92% positivity *vs* 78% for FBC; Ruddy and Winer, 2013), and has some differences in genetic (Johansson *et al*, 2011; Kornegoor *et al*, 2012; Piscuoglio *et al*, 2016), transcriptomic (Callari *et al*, 2011; Johansson *et al*, 2012) and protein expression profiles (Shaaban *et al*, 2012; reviewed in Deb *et al*, 2016). Incidence trends in terms of geographical location and impact of patient age for both diseases are broadly similar (Kreiter *et al*, 2014). These studies have not given insights that suggest that different treatment approaches are appropriate, either in terms of which prognostic or predictive markers might be useful, or which therapies should be used. Also, there are no prospective randomised controlled trials for MBC that could inform treatment decisions (Bratman *et al*, 2012). Consequently, MBC management is based on data from FBC. One key difference, however, is that the vast majority of MBC patients undergo mastectomies (Korde *et al*, 2010), whereas breast-conserving surgery is prevalent for FBC. It is worth noting that this difference is not based on evidence concerning treatment outcomes, rather on practical issues relating to the size of breast tissue. Adjuvant therapies, including

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radiotherapy (Ruddy and Winer, 2013), tamoxifen (Ribeiro and Swindell, 1992; Fogh *et al*, 2011) and chemotherapy (Korde *et al*, 2010), are essentially the same.

The eukaryotic translation initiation factor 4E (eIF4E) is a key component of the translational machinery and has two specific functions. Firstly, it recognises and binds to mRNA caps within the cytoplasm allowing initiation of cap-dependent translation (Sonenberg, 2008), the mechanism responsible for most protein synthesis (Gray and Wickens, 1998). Secondly, eIF4E binds to some mRNAs within the nucleus and regulates their nuclear export (Culjkovic *et al*, 2005, 2007). Activity of eIF4E is controlled largely by the eIF4E-binding proteins (4E-BPs), of which there are three, although only 4E-BP1 and 2 have been studied in any detail. Eukaryotic initiation factor 4E function is inhibited when bound by 4E-BPs (Matsuo *et al*, 1997), but this interaction is itself regulated by a series of sequential phosphorylations to the 4E-BPs mediated via the mTORC1 complex (Gibbons *et al*, 2009). Phosphorylated 4E-BPs are unable to bind to eIF4E. Thus, eIF4E activity is defined by a subtle balance of expression levels of eIF4E and the 4E-BPs, and the phosphorylation status of the 4E-BPs (Coleman *et al*, 2009). Activity of eIF4E is frequently increased in a wide range of cancers (De Benedetti and Graff, 2004), resulting in enhanced translation (and potentially nuclear export) of a subset of mRNAs that contains many cancer-related transcripts. In FBC, eIF4E is frequently expressed at higher levels in breast cancers compared with normal or benign breast tissue (Kerekatte *et al*, 1995; Norton *et al*, 2004) and higher levels of eIF4E are associated with poorer prognoses (Li *et al*, 2002; Byrnes *et al*, 2006). In addition, higher levels of the phosphorylated form of 4E-BP1 (p4E-BP1) are also seen in FBC compared with normal and benign tissue (Zhou *et al*, 2004), and these levels are positively associated with grade, lymph node metastasis and disease recurrence (Rojo *et al*, 2007). Our own work has demonstrated that combined analysis of expressions of eIF4E, 4E-BP1, 4E-BP2 and p4E-BP1 predicts breast cancer survival in females and represents an estimate of eIF4E activity (Coleman *et al*, 2009). The influential role that eIF4E plays in neoplasia has made it an attractive anticancer drug target. Therapeutic approaches that have been explored include knock-down of eIF4E expression (Graff *et al*, 2007; Hong *et al*, 2011), blocking of eIF4E-cap binding (Assouline *et al*, 2009; Pettersson *et al*, 2011), inhibition of eIF4E phosphorylation in an effort to reduce its activity (Wheater *et al*, 2010), and – most commonly – inhibition of mTORC1 activity leading to 4E-BP hypophosphorylation and inhibitory binding to eIF4E (Chan *et al*, 2005; Wazir *et al*, 2014). It should be noted that inhibition of mTORC1, or more generally the mTOR kinase component of this complex, clearly has anticancer influence that is independent of 4E-BP1 through other targets of the complex (Laplante and Sabatini, 2009), and therefore this approach is in no way equivalent to direct targeting of eIF4E. Currently nothing is known about the prognostic relevance of eIF4E and the 4E-BPs in MBC, and there is no evidence base from which novel eIF4E-directed therapies might be considered in this disease; our aim was to perform the first investigation of the importance of these molecules in this cancer type.

MATERIALS AND METHODS

Patients and tissue microarrays. Ethical approval was obtained from Leeds (West) (ref 06/Q1205/156) and Leeds (East) Research Ethics Committees (ref 05/Q1206/136). Archival resection samples of invasive breast cancers from MBC patients ($n=337$) and associated clinical and pathological data were collected from the United Kingdom (157; 46.6%), Italy (50; 14.8%), Hungary (41; 12.2%), Poland (30; 9.5%), Canada (50; 14.8%) and Nigeria (9; 2.7%). Clinico-pathological characteristics are shown in Table 1. Survival data were available for 187 cases. Tissue microarrays (TMAs) were constructed from tissues; this process has been described in detail previously (Shaaban *et al*, 2012). In summary, H + E-stained tumour sections were reviewed by specialist breast consultant histopathologists (RAM-S, AMH, Dr Abeer Shaaban (Queen Elizabeth Hospital, Birmingham, UK)) in order to confirm diagnoses and select representative areas of invasive carcinoma from which TMA cores would be taken. Tissue microarrays were constructed of duplicate or triplicate 0.6mm tumour cores from each individual case. Seven TMA blocks were used for the cohort, each including a perimeter wall of non-breast tissue (liver, sheep lung, placenta and brain) to minimise edge effects and to provide internal controls.

Table 1. Clinical and pathological features of the cohort

Characteristics	Number (%) $n=337$
Histological type	
Ductal no-special type	275 (81.6)
Papillary/encysted papillary	17 (5.1)
Mucinous	11 (3.3)
Lobular	3 (0.9)
Other special type	5 (1.5)
Mixed	8 (2.4)
Unknown	8 (2.4)
Tumour grade	
1	44 (13.1)
2	158 (46.9)
3	121 (35.9)
Ungraded	14 (4.2)
Tumour size	
1 (<2 cm)	70 (20.8)
2 (2–5 cm)	65 (19.3)
3 (>5 cm)	14 (4.2)
Unknown	188 (55.8)
LN status	
At least 1 positive node	112 (33.2)
No positive nodes	101 (30.0)
Unknown	124 (36.8)
ER status	
Positive (Allred score >2)	238 (70.6)
Negative	52 (15.4)
Unknown	47 (13.9)
PR status	
Positive (Allred score >2)	238 (70.6)
Negative	48 (14.2)
Unknown	51 (15.1)

Abbreviations: ER = oestrogen receptor alpha; LN = lymph node; PR = progesterone receptor.

Immunohistochemistry. Immunohistochemistry was carried out as previously described (Coleman *et al*, 2009). In summary, 5 μm sections were taken from blocks, and were deparaffinised and re-hydrated. Appropriate antigen retrieval (see below) was performed and sections were treated with 1% hydrogen peroxide–methanol to inhibit endogenous peroxide activity. Sections were stained overnight with primary antibodies (see below) diluted in antibody diluent solution (Invitrogen, Carlsbad, CA, USA). Signals were visualised using the DAB based Envision System (Dako, Glostrup, Denmark). All case TMAs, and a control TMA of FBCs, were stained for each antibody as a single batch. Female cores served as positive and negative controls. Antibodies, dilutions and antigen retrieval: eIF4E (moue monoclonal sc9976; Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1 : 100; boiled for 2 min in a pressure cooker in antigen unmasking solution, Vector, Burlingame, CA, USA); 4E-BP1 (rabbit polyclonal 9452;

Cell Signalling Technology (Danvers, MA, USA); 1 : 100; no antigen retrieval); 4E-BP2 (rabbit polyclonal 2845; Cell Signalling Technology); 1 : 100; 12 min full power microwave in pH6 citrate buffer); p4E-BP1 Thr37/46 (rabbit polyclonal 2855; Cell Signalling Technology; 1 : 25; 12 min full power microwave in pH6 citrate buffer). The specificities of these antibodies have been validated previously and they have all been used successfully for immunohistochemistry in breast tissue previously (Zhou *et al*, 2006; Coleman *et al*, 2009; Satheesha *et al*, 2011).

Scoring and statistics. Stained TMAs were digitally scanned (Aperio, Oxford, UK), and cores were scored independently by two consultant histopathologists (RAM-S and CDS) from the same digital images. Cytoplasmic and nuclear immunoreactivity was separated and given individual scores. The scoring system incorporated scores for staining intensity in tumour cells (0 no staining, 1 weak staining, 2 moderate staining and 3 strong staining) added to scores for proportions of tumour cells staining positively (1 $<$5%, 2 6–25%, 3 26–75% and 4 $>$75%), giving totals of either 0 or from 2 to 7, as has been used previously for these antigens (Zhou *et al*, 2006; Coleman *et al*, 2009). Analyses were performed in SPSS (SPSS, Chicago, IL, USA) unless stated otherwise. Correlations between antigen expression scores and clinical factors were examined by calculating Spearman rho correlation coefficients. Associations with disease recurrence and survival were analysed by Kaplan–Meir survival curves and log rank tests following ROC curve analysis to dichotomise the expression scores into low and high expression appropriately. Kappa calculations were performed using Analyse-it for Excel (Microsoft, Redmond, WA, USA). All tests were two-sided.

RESULTS

eIF4E, 4E-BP1, 4E-BP2 and p4E-BP1 expression varies widely in MBC. Tissue microarrays (TMAs) containing duplicate or triplicate samples from 337 male breast tumours were stained using immunohistochemistry to analyse expression of eIF4E, 4E-BP1, 4E-BP2 and p4E-BP1. Cores were scored by two independent histopathologists in terms of expression intensity and proportions of cells staining positively. To take into account the potentially different roles of these protein species in different cellular compartments (Culjkovic *et al*, 2006; Sonenberg, 2008), cytoplasmic and nuclear immunoreactivity were separated and given individual scores. Scores from the two histopathologists were highly concordant, demonstrating robust and reproducible scoring; quadratic weighted kappa statistics were 0.85–0.96 for cytoplasmic scores and 0.74–0.95 for nuclear (depending on antigen; see Supplementary Table S1). Core loss, an expected and documented occurrence in TMA-based research (Parsons and Grabsch, 2009), or lack of tumour cells meant that staining was not assessable in some cases; however a mean of 2.3 cores was successfully scored for each case for each antibody. We analysed variability in scores between multiple cores representing individual tumours in order to assess potential heterogeneity within individual tumours and therefore the representative nature of TMA cores. Spearman's rho correlation coefficients for duplicate scores for each tumour and antigen were all 0.79 ($P<0.001$) or over, demonstrating that there was relatively little heterogeneity in marker expression within individual tumours and that TMA-based analyses were appropriate. Having determined that inter-scorer and core-to-core variability were low, we took mean values of all the scores available for each case/antigen/subcellular location to create single scores for further analysis. Representative staining and the frequency distributions of these scores (rounded to the nearest whole number) are shown in Figure 1. The full range of expression patterns was seen for each antigen, ranging from no detectable expression to strongly expressed in more than 75% of tumour cells. The distributions of cytoplasmic and nuclear scores were broadly similar for each antigen, and expressions in the two compartments were strongly associated (Spearman's rho correlation coefficients 0.85–0.95, $P<0.0008$), suggesting that separating the two scores gave relatively little additional information.

Expression of eIF4E correlates weakly with ER status in MBC. Associations between marker expression and established prognostic factors were examined. The factors tested were: (1) histological tumour grade (1, 2 or 3); (2) tumour size (categorised as 2 cm or less, $>$2 cm but $\leqslant$ 5 cm, or $>$5 cm); (3) lymph node status (negative or positive); and (4) oestrogen receptor alpha (ERα) status (negative (Allred 0 or 2) or positive (Allred $>$2)). Spearman's rho correlation coefficients (r) were calculated for each potential association. The only associations with Spearman's coefficients $>$0.2, which is weak at best, were both

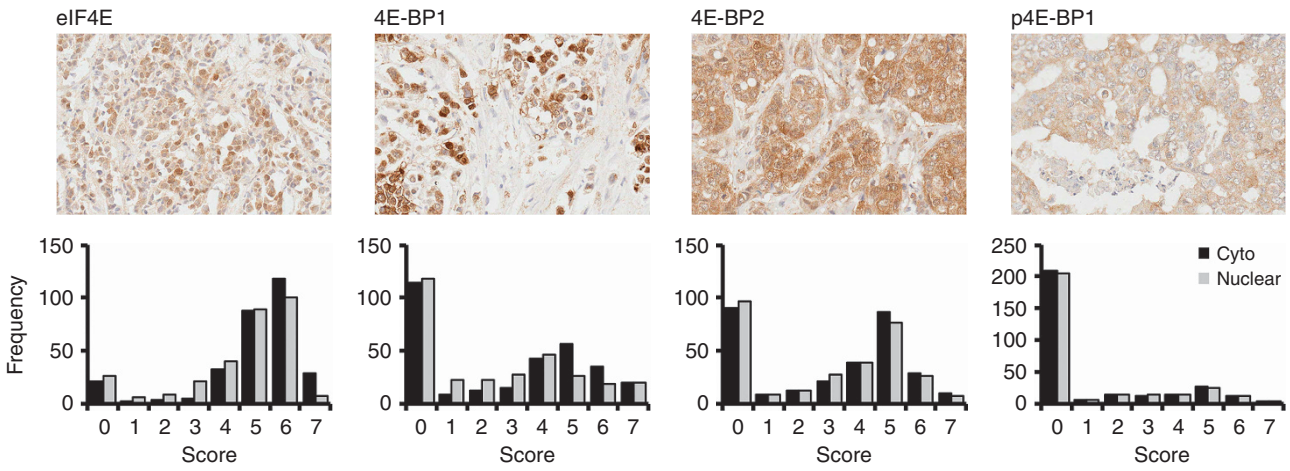

Figure 1. Male breast cancer (MBC) has a full range of expression patterns for eIF4E, 4E-BP1, 4E-BP2 and p4E-BP1. Tissue microarrays containing multiple tumour cores from 337 MBCs were stained as indicated using immunohistochemistry. Cytoplasmic and nuclear expressions in tumour cells were assessed as 0 (negative) or 2–7 (positive, increasing intensity/proportion of positive cells). Representative positive staining is shown at the top of the panel for each antigen. Images shown were scored for cytoplasmic (c) and nuclear (n) expression as follows: eIF4E – c 7, n 6; 4E-BP1 – c 4, n 5; 4E-BP2 – c 5, n 0; p4E-BP1 – c 6, n 3. Frequency distributions of cytoplasmic (black) or nuclear (grey) expression across the cohort are shown below. Mean scores for each case were determined and are represented rounded to the nearest whole number.

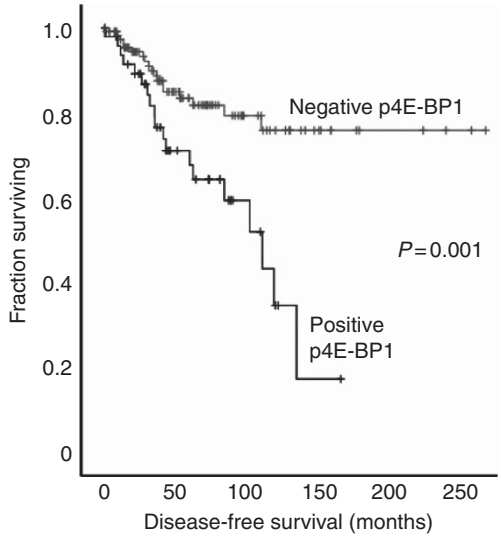

Figure 2. Expression of p4E-BP1 is significantly associated with disease-free survival in male breast cancer ($P=0.001$). Kaplan–Meier survival analyses for patient groups with tumours with either no detectable (negative; grey line) or any detectable (positive; black line) expression of p4E-BP1.

cytoplasmic and nuclear eIF4E expression being positively associated with ERα status ($r=0.231$ and 0.202, respectively; $P<0.002$).

Expression of p4E-BP1, but not eIF4E, is strongly associated with MBC survival. Kaplan–Meier survival analyses were performed to determine whether expression of the markers was significantly related to DFS. Cutoffs were applied to dichotomise patients into two groups based on low or high expression of each marker. These cutoffs were defined objectively using receiver operator curve analyses (Zlobec *et al*, 2007) to give the best balance between sensitivity and specificity for prediction of the relevant clinical outcome (i.e. breast cancer recurrence). The cutoff values are shown in Supplementary Table S2. Kaplan–Meier survival analyses were performed and log rank tests were used to assess the significance of relationships. A stringent value of $P<0.003$ was defined as indicating significance, after Bonferroni correction for multiple tests from an initial value of $P<0.05$. Only cytoplasmic expression of p4E-BP1 demonstrated a significant relationship with survival (Figure 2), with patients with low p4E-BP1 expression having a longer DFS than those with high expression (215 *vs* 95 months, $P=0.001$). It is important to note that the cutoff to dichotomise p4E-BP1 expression was 0.83, meaning that tumours in the two groups were those without detectable p4E-BP1 (negative) or those with any detectable expression (positive). Cytoplasmic and nuclear expression of 4E-BP1, and – surprisingly – nuclear expression of 4E-BP2, also showed trends towards significant relationships with survival, although these fell short of our stringent significance test. Expression of eIF4E itself showed no such trend (Supplementary Table S3).

Estimated eIF4E activity is not associated with MBC survival. We have previously demonstrated in FBC that assessments of expression of these markers could be combined to estimate eIF4E activity, an estimated value that was significantly associated with survival (Coleman *et al*, 2009). Activity (referred to as '*z*') was estimated as *X*–BP1/4 + pBP1/2–BP2/4, where *X* represents the eIF4E score, BP1 the 4E-BP1 score, BP2 the 4E-BP2 score and pBP1 the p4E-BP1 score. This estimate was determined for these MBC cases using the cytoplasmic scores, and receiver operator curve analysis was performed to determine a suitable cutoff to split the cohort in groups with high and low *z* scores. Kaplan–Meier survival analyses were performed. Estimated eIF4E activity was not significantly associated with DFS (Supplementary Table S3).

Table 2. Cytoplasmic p4E-BP1 is significantly associated with survival in univariate and multivariate regression analysis

	Univariate analysis		Multivariate analysis	
	Hazard ratio	*P*-value	Hazard ratio	*P*-value
Cytoplasmic p4E-BP1	3.073	0.001	8.755	<0.0005
Tumour size	1.963	0.048	2.923	0.016
Tumour grade	0.704	0.165	0.432	0.129
LN status	1.494	0.326	4.976	0.018
ER status	1.792	0.277	1.176	0.788

Abbreviations: ER = oestrogen receptor alpha; LN = lymph node.

Cytoplasmic p4E-BP1 is significantly associated with survival in multivariate analysis. Multivariate regression was performed to assess whether cytoplasmic expression of p4E-BP1 was an independent prognostic factor with regard to DFS. The other variables put into the model were the currently used prognostic factors of grade, tumour size, lymph node status and ERα status. Both cytoplasmic p4E-BP1 expression and tumour size were significantly associated with DFS on univariate and multivariate analyses, although cytoplasmic p4E-BP1 expression consistently showed the greater significance and the more informative hazard ratio (Table 2).

DISCUSSION

This study is the first in which expressions and prognostic relevance of eIF4E and the 4E-BPs have been examined in MBC. Our analysis involved one of the largest MBC cohorts assembled ($n=337$) and thorough immunohistochemical analyses with multiple tissue samples per case, very robust histopathological scoring and well-validated antibodies. It is also worth noting that our work is the first in any cancer to separately investigate the prognostic worth of eIF4E and its regulatory molecules in cytoplasmic and nuclear compartments, in accordance with their different reported roles in these locations (De Benedetti and Graff, 2004; Siddiqui and Borden, 2012). We found expression in these compartments to be tightly correlated, and separate prognostic insights were not gained from the compartment analysis. Interestingly, some individual cases with prominent nuclear only or cytoplasmic only expression were noted, suggesting that subcellular regulation may take place in some circumstances; however, cases were infrequent and analysis of their common clinicopathological features was flawed on this basis. A rare precedent for separating different subcellular localisation of these molecules in cancer is, remarkably, also in the context of MBC. Nuclear and cytoplasmic distributions of p4E-BP1 have been reported previously in 56 familial MBCs, showing expression in the two compartments to be highly associated and positive in 52 and 55% of cases, respectively (slightly more than we find) (Deb *et al*, 2013).

Surprisingly, and in marked contrast to FBC (Coleman *et al*, 2009), no association was found between eIF4E expression and survival. Expression of eIF4E has been associated with prognosis in a wide range of cancers (De Benedetti and Graff, 2004), but there are specific cancers where this is not the case, for example, in acute myeloid leukaemia (Green *et al*, 2012) or osteosarcoma (Osborne *et al*, 2011). In addition, there is likely to be a publication bias against such findings, so it may be that this lack of association is

more common than currently appreciated. Strikingly, however, we identified a strong prognostic association for p4E-BP1, with any detectable p4E-BP1 expression correlated with poor survival in both univariate and multivariate analyses (Figure 2 and Table 2). This association was far stronger than previously found in FBC (Coleman *et al*, 2009). 4E-BP1 phosphorylation breaks 4E-BP1's inhibitory interaction with eIF4E resulting in increased eIF4E activity (De Benedetti and Graff, 2004); therefore, one might expect that p4E-BP1 could only be functionally associated with prognosis through the eIF4E pathway. Yet, here we show that the wide variations in expression of eIF4E itself, or in estimated eIF4E activity, do not impact on prognosis (Supplementary Tables S2 and S3), rendering this expectation incompatible with our data. We interpret this to suggest that p4E-BP1 is acting as a biomarker for functionally relevant activity of the upstream kinase, the mTORC1 complex, rather than having a direct functional impact on prognosis itself. In support of this, it is well established that levels of p4E-BP1 correlate with mTORC1 activity in various contexts, and accordingly p4E-BP1 has frequently been used as a pharmacodynamic marker for mTORC1 activity in trials of mTORC1-targeting therapeutics (Tabernero *et al*, 2008; Spunt *et al*, 2011).

The kinase within the mTORC1 complex is mTOR, upregulation of which is associated with development of many cancers (Shaw and Cantley, 2006). The mTORC1 complex acts on a large number of different molecular substrates (Laplante and Sabatini, 2009; Hsu *et al*, 2011), although the functional importance of two have been studied in considerably more detail than the others with regard to cancer: 4E-BP1 and S6 kinase 1 (S6K1). In MBC we believe that 4E-BP1 may not be a functionally relevant substrate; therefore, it seems likely that deregulated mTOR acts at least in part through S6K1 and its downstream effectors. Phosphorylated (activated) S6K1 can induce oncogenic increases in overall protein translation, and changes in sterol, lipid and mitochondrial metabolism via a variety of complex signalling pathways (Alayev and Holz, 2013). Expression levels of both eIF4E and mTOR have been noted in a previous analysis of gene expression profiles in MBC ($n = 37$) as compared with FBC (Callari *et al*, 2011). Both proteins were found to be more highly expressed in MBC than in FBC, and the authors commented that the eIF4E pathway may therefore present an attractive therapeutic target in MBC. Our findings impact on this suggestion, in that we find eIF4E itself to be unrelated to prognosis, while we infer that mTOR activity within the mTORC1 complex may well relate to prognosis. Thus, our data do not support use of therapies directed at eIF4E itself, such as knockdown of eIF4E expression (Graff *et al*, 2007; Hong *et al*, 2011), or function (Assouline *et al*, 2009; Wheater *et al*, 2010; Pettersson *et al*, 2011), but do support potential use of therapies directed at the upstream kinase, mTOR.

These findings may delineate potential differences in appropriate treatments between FBC and MBCs. For example, the eIF4E-directed therapies LY2275796 (anti-sense oligonucleotides directed against eIF4E) and ribavirin (which reduces eIF4E-dependent translation) have shown some promise in preclinical or clinical trials (Hong *et al*, 2011; Pettersson *et al*, 2015), and are undergoing evaluation for FBC. Our data suggest that these may have limited efficacy in MBC since eIF4E activity appears relatively unimportant in determining prognosis in this disease. However, by contrast, the growing list of therapies targeting mTOR (Sun, 2013), such as everolimus or temsirolimus that have already shown promise in FBC trials (Baselga *et al*, 2012; Wolff *et al*, 2013), may well be suitable therapies in both female and male cancers. Interestingly, there is a single case report describing a favourable response of an MBC patient to temsirolimus (Katayama *et al*, 2013), but unfortunately it seems unlikely that an MBC trial will take place due to the rarity of the disease overall. A further issue would be that fewer than 50% of MBC cases expressed detectable p4E-BP1 in our data (Figure 1), and therefore only a minority may potentially be suitable for this approach. Nevertheless, we conclude that mTOR-targeted therapies may be worth considering in p4E-BP1-positive MBC.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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