Published OnlineFirst December 16, 2016; DOI: 10.1158/1078-0432.CCR-16-1952

Biology of Human Tumors

Clinical Cancer Research

A Case-Matched Gender Comparison Transcriptomic Screen Identifies eIF4E and eIF5 as Potential Prognostic Markers in Male Breast Cancer

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Abstract

Purpose: Breast cancer affects both genders, but is understudied in men. Although still rare, male breast cancer (MBC) is being diagnosed more frequently. Treatments are wholly informed by clinical studies conducted in women, based on assumptions that underlying biology is similar.

Experimental Design: A transcriptomic investigation of male and female breast cancer was performed, confirming transcriptomic data *in silico*. Biomarkers were immunohistochemically assessed in 697 MBCs ($n = 477$, training; $n = 220$, validation set) and quantified in pre- and posttreatment samples from an MBC patient receiving everolimus and PI3K/mTOR inhibitor.

Results: Gender-specific gene expression patterns were identified. eIF transcripts were upregulated in MBC. eIF4E and eIF5 were negatively prognostic for overall survival alone (log-rank $P = 0.013$; HR = 1.77, 1.12–2.8 and $P = 0.035$; HR = 1.68, 1.03–2.74, respectively), or when coexpressed ($P = 0.01$; HR = 2.66, 1.26–5.63), confirmed in the validation set. This remained upon multivariate Cox regression analysis [eIF4E $P = 0.016$; HR = 2.38 (1.18–4.8), eIF5 $P = 0.022$; HR = 2.55 (1.14–5.7); coexpression $P = 0.001$; HR = 7.04 (2.22–22.26)]. Marked reduction in eIF4E and eIF5 expression was seen post BEZ235/everolimus, with extended survival.

Conclusions: Translational initiation pathway inhibition could be of clinical utility in MBC patients overexpressing eIF4E and eIF5. With mTOR inhibitors that target this pathway now in the clinic, these biomarkers may represent new targets for therapeutic intervention, although further independent validation is required. *Clin Cancer Res; 23(10); 2575–83. ©2016 AACR.*

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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doi: 10.1158/1078-0432.CCR-16-1952

Published OnlineFirst December 16, 2016; DOI: 10.1158/1078-0432.CCR-16-1952

Translational Relevance

Genomic and transcriptomic analysis of four independent male breast cancer datasets identified upregulation of translational initiation pathway genes. eIF4E and eIF5 were independent predictors of survival, either alone or when coexpressed. Samples from a patient receiving a combination of agents targeting this pathway suggest this pathway may be tractable.

Introduction

The need for more refined therapeutic treatments for male breast cancer (MBC) is evidenced by a steady stream of publications highlighting gender-specific differences using IHC (1–5), genetics (6–11), and more recently, epigenetics (12–15). Of note, although MBC is similar histologically to female breast cancer (FBC), with the same panel of biomarkers used to guide treatment and prognosis, more rigorous interrogation of the underlying genetics shows heterogeneity in MBC as recognized in FBC where molecular profiling has identified different subgroups that correlate with varying clinical outcomes. Gene expression analysis of MBC is more limited. Nevertheless, genetic disparity has been reported, notably genes involved in extracellular matrix remodeling, metabolism, and protein synthesis via genes involved in translational initiation, including *eIF4E* (10), which are often upregulated in MBC compared with FBC. Further work has identified two distinct subgroups of MBC, termed luminal M1 and luminal M2, which differed from molecular subtypes seen in FBC (9). This work also reported that *N*-acetyltransferase-1, a gene thought to be involved in drug metabolism, was a prognostic marker for MBC (9). Subsequent to this, Johansson and colleagues documented differential driver genes in MBC versus FBC (16). Most recently, a distinct repertoire of genetic alterations was reported in MBC, cautioning the application of FBC data to therapeutic application in MBC (11). Genomic and immunohistochemical examination of a single MBC patient with recurrent disease showed a change in hormone receptor expression in the postprogression sample, with little change at the genomic level, while receiving a combination of BEZ235/everolimus (17).

Taking advantage of our large collection of MBC samples, we aimed to generate gene expression profiles of matched MBC and FBC samples and assess immunohistochemically whether differences in specific biomarkers affected clinical outcome in men using a training set of 477 and a validation set of 220 cases. Finally, we analyzed expression of these biomarkers in pre- and posttreatment samples from an MBC patient who received a combination of the PI3K/mTOR inhibitors BEZ235 and everolimus (17).

Materials and Methods

Ethical approval and patient material

Leeds (East) Research Ethics Committee (06/Q1205/156; 15/YH/0025) granted ethical approval. For gender comparison transcriptomics, cases were matched for age, size, nodal, and survival status. Formalin-fixed paraffin-embedded male ($n = 15$) and female ($n = 10$) primary invasive ductal carcinoma [estrogen receptor (ER) positive, HER2 negative, node negative] were identified from histopathology archives. An additional 3 male and 3 female frozen cases were used to confirm gene expression. A training set of 477 MBCs represented on tissue microarrays (TMA; $n = 446$, constructed as described in ref. 1) and 31 full-faced sections, plus a validation set [220 cases on TMAs (9)], was used in IHC. Patient characteristics are shown in Table 1. Details on the datasets used in the explorative and validation phases are provided (Supplementary Fig. S1). Cases were pseudo-anonymized and data analyzed anonymously.

Gene expression

Extracts from five 10-μm sections were applied to Almac Diagnostics Breast Cancer DSA platform representing 21,808 genes, according to in-house protocols (18). Three MBC samples failed QC and were excluded from further analysis. Genes that were significantly differentially expressed between genders were calculated from Almac-normalized and transformed data with FDR threshold of 5% and a fold change significance of 1%. Representative heatmaps were generated from resulting expression data using hierarchical clustering and Pathway Ingenuity Analysis to identify gender-specific gene expression. The microarray data are available on ArrayExpress (www.ebi.ac.uk/arrayexpress, accession number E-MTAB-4040). The Oncomine platform was used for further data mining.

IHC

REMARK criteria were employed (19). IHC was conducted as described previously, using well-validated antibodies (20), including eIF1 (Abcam; ab118979, 1:200), eIF2 (Abcam; ab32157, 1:150), eIF3 (Abcam; ab171419, 1:150), eIF4E (Santa

Table 1. Clinicopathologic data for the MBC training and validation sets

Characteristics	Training set	Validation set
Mean age (range)	66 (30–97)	70 (23–98)
Mean follow-up, years (range)	3.9 (0.08–24.5)	4.6 (0.04–15)
Treatment	Various combinations of adjuvant hormonal, chemo, and radiotherapy	
Histology	**Number (%)**	**Number (%)**
Invasive	419 (88)	130 (59)
DCIS	7 (1)	4 (2)
Mixed	15 (3)	47 (21)
Unknown	36 (8)	39 (18)
Grade		
1	50 (10)	15 (7)
2	193 (41)	98 (44)
3	147 (31)	85 (39)
Unknown	87 (18)	22 (10)
Lymph node		
+	134 (28)	78 (35)
−	147 (31)	83 (38)
Unknown	196 (41)	59 (27)
ERα		
+	404 (85)	193 (88)
−	30 (6)	9 (4)
Unknown	43 (9)	18 (8)
PR		
+	352 (74)	160 (73)
−	74 (15)	41 (19)
Unknown	51 (11)	19 (9)
HER2		
+	6 (1)[a]	18 (8)[a]
−	291 (65	157 (71)
Unknown	149 (34)	45 (20)

Abbreviation: DCIS, ductal carcinoma *in situ*.
[a]Confirmed by FISH/CISH.

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Cruz Biotechnology; sc-9976, 1:400), and eIF5 (Abcam; ab32443, 1:300). Cases were batch stained for each antibody with recommended controls. TMAs were digitized (×40, Leica-Aperio AT2 ScanScope scanner; Leica Biosystems). Each TMA core was viewed using in-house software and assessed semiquantitatively for each biomarker, taking account of staining intensity and percentage of tumor cells. Overall scores were averaged from either duplicate or triplicate cores that represented a case. Staining was generally cytoplasmic; our group has shown that nuclear staining is seen occasionally but is not of prognostic value (20); therefore, only cytoplasmic staining was considered. Scoring criteria were determined from previously reported studies (20, 21). Cases were scored by MPH with coscoring of 10% (C.A.B. Suleman, trainee histopathologist), overseen by A.M. Shaaban, specialized breast consultant histopathologist. Where disagreement was reported (score >2; $n = 5$), cases were rereviewed to reach consensus. Excellent strength of agreement was observed between scorers using interclass correlation coefficients (eIF1 0.911 [95% confidence interval (CI), 0.769–0.944], eIF2 0.846 (95% CI, 0.736–0.910), eIF4E 0.882 (95% CI, 0.755–0.913), and eIF5 0.865 (95% CI, 0.769–0.922). Scores were indeterminable in 49 cases due to core loss/exhaustion during processing, well-recognized with TMAs.

Analysis of eIF4E and eIF5 on a single patient progression series treated with PI3K/mTOR inhibitors

Pre- and posttreatment biopsies were obtained from a 66-year-old Caucasian male diagnosed in 2006 with ER^+, progesterone receptor positive (PR^+), $HER2^-$ infiltrative papillary breast cancer whose clinical history has been reported (17). Following mastectomy, he received adjuvant tamoxifen but developed a contralateral grade 3 ER^+, PR^+, $HER2^-$ infiltrative ductal carcinoma 2 years later (pretreatment sample). Standard adjuvant chemotherapy commenced, with 5 weeks of

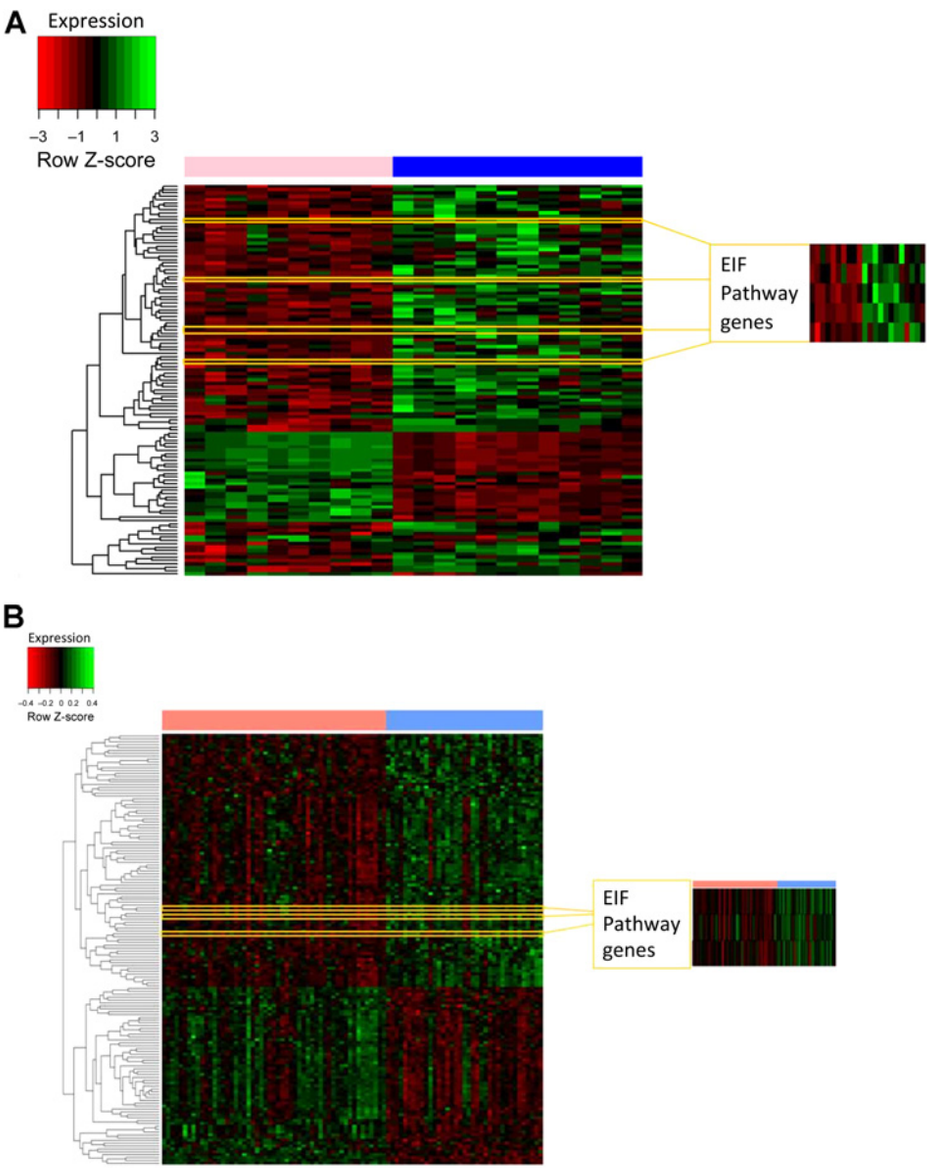

Figure 1.
Identification of eIF pathway upregulation in MBC by hierarchical clustering and validation in an external dataset. **A,** Heatmap showing gender-specific hierarchical clustering of differentially expressed genes in female (pink) and male (blue) breast cancers with exploded view of eIF genes, which were significantly overexpressed in MBC on the right ($P < 0.0001$; eIF pathway genes and $P = 0.016$; FDR). **B,** Hierarchical clustering of a reanalysis of the Callari et al. dataset (10) similarly identified members of the eIF family were overexpressed in MBC as shown in the exploded view on the right. Green, overexpression; red, underexpression.

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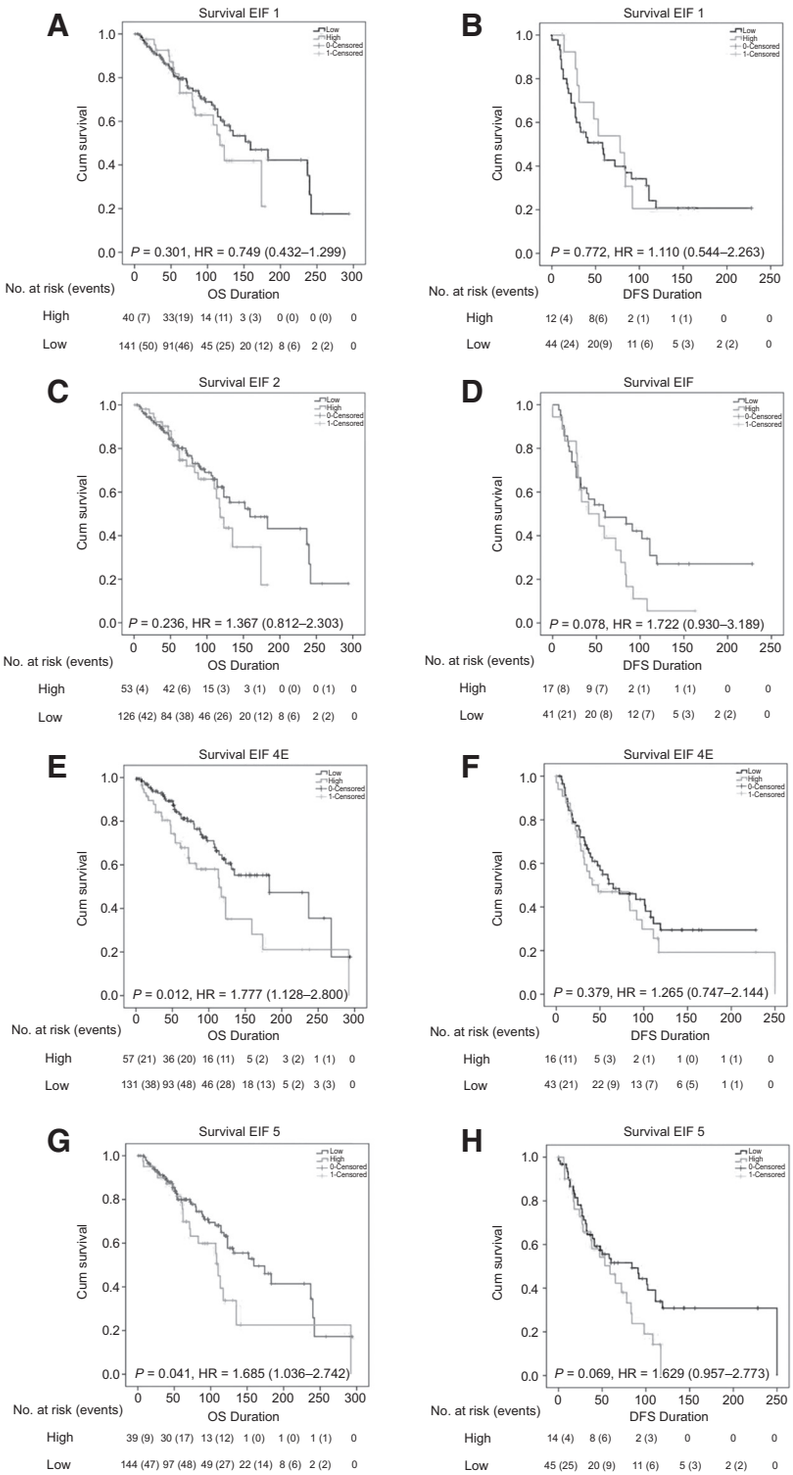

Figure 2.

The effect of eIF expression on DFS and OS in MBC by Kaplan–Meier survival analysis. **A–H,** Effects on OS are shown in **A**, **C**, **E**, and **G** and DFS in **B**, **D**, **F**, and **H**. **A** and **B** = eIF1; **C** and **D**; eIF2; **E** and **F** = eIF4E; and **G** and **H** = eIF5. Gray line, high expression; black line, low expression, dichotomized by ROC analysis and analyzed by log-rank test.

radiotherapy and subsequent adjuvant letrozole. Thirteen months later, he developed multiple nodal and bilateral lung metastases and was switched to a schedule of vinorelbine plus capecitabine every 3 weeks. Following disease stabilization, he received fulvestrant. After 8 months, node progression was noted, and the patient was switched to BEZ235 (200 mg orally, twice daily) plus subtherapeutic everolimus (2.5 mg orally, weekly). Aside from a skin rash, this was well tolerated, and stable disease was maintained for a further 18 months after which a nodal metastasis developed (posttreatment sample). eIF4E and eIF5 expression was assessed immunohistochemically in the pre- and posttreatment samples, as described above

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Table 2. Univariate and multivariate analysis of eIF4E and eIF5 expression in MBC

Univariate analysis (all biomarkers)

	Training set		Validation set		Combined dataset	
Variable	**HR (CI)**	***P***	**HR (CI)**	***P***	**HR (CI)**	***P***
Grade	1.590 (1.007–2.511)	0.047	1.116 (0.849–1.466)	0.432	1.252 (1.006–1.557)	0.044
Age	1.055 (1.032–1.079)	0.000002	1.004 (1.002–1.005)	0.000017	1.005 (1.003–1.006)	2.1E−10
Size (>20 mm)	1.006 (0.997–1.014)	0.209	1.428 (0.990–2.059)	0.057	1.146 (1.080–2.016)	0.014
Node positivity	1.549 (0.948–2.532)	0.081	1.150 (1.094–1.209)	4.4E−09	1.695 (1.252–2.295)	0.001
eIF4E	1.777 (1.128–2.800)	0.013	1.564 (1.028–2.378)	0.037	2.196 (1.634–2.952)	1.4E−07
eIF5	1.685 (1.036–2.742)	0.035	1.674 (1.003–2.793)	0.049	1.347 (0.944–1.922)	0.101
Coexpression	2.664 (1.260–5.633)	0.01	2.228 (1.093–4.542)	0.027	2.776 (1.683–4.579)	0.00006

Multivariate analysis (EIF4E)

	Training set		Validation set		Combined dataset	
Variable	**HR (CI)**	***P***	**HR (CI)**	***P***	**HR (CI)**	***P***
Grade	1.002 (0.583 1.721)	0.995	1.106 (0.826–1.483)	0.498	1.169 (0.902–1.515)	0.237
Age	1.052 (1.017–1.088)	0.003	1.003 (1.002–1.005)	0.0001	1.004 (1.002–1.006)	0.000005
Size (>20 mm)	1.008 (0.997–1.019)	0.173	1.223 (0.828–1.805)	0.312	1.203 (0.885–1.692)	0.290
Node positivity	1.445 (0.739–2.822)	0.282	1.131 (1.072–1.193)	0.000006	1.621 (1.150–2.286)	0.006
eIF4E	2.380 (1.179–4.805)	0.016	1.333 (0.866–2.052)	0.192	2.297 (1.576–30262)	0.00001

Multivariate analysis (EIF5)

	Training set		Validation set		Combined dataset	
Variable	**HR (CI)**	***P***	**HR (CI)**	***P***	**HR (CI)**	***P***
Grade	1.075 (0.606–1.907)	0.805	1.065 (0.787–1.441)	0.683	1.101 (0.843–1.437)	0.482
Age	1.070 (1.033–1.107)	0.0001	1.003 (1.001–1.005)	0.002	1.004 (1.002–1.005)	0.0001
Size (>20 mm)	1.008 (0.997–1.019)	0.138	1.248 (0.833–1.870)	0.282	1.294 (0.922–1.117)	0.136
Node positivity	1.813 (0.911–3.610)	0.09	1.134 (1.073–1.198)	0.000008	1.621 (1.150- 2.286)	0.007
eIF5	2.552 (1.142–5.702)	0.022	1.528 (0.881–2.650)	0.131	2.267 (1.576–3.262)	0.044

Multivariate analysis (coexpression of EIF4E and EIF5)

	Training set		Validation set		Combined dataset	
Variable	**HR (CI)**	***P***	**HR (CI)**	***P***	**HR (CI)**	***P***
Grade	0.391 (0.137–1.114)	0.079	1.692 (0.858–3.336)	0.129	0.865 (0.508–1.472)	0.592
Age	1.039 (0.992–1.088)	0.104	1.003 (1.001–1.006)	0.01	1.004 (1.002–1.007)	0.001
Size (>20 mm)	1.008 (0.991–1.026)	0.34	2.530 (1.170–5.472)	0.018	1.869 (1.040–30360)	0.037
Node positivity	2.927 (0.953–8.992)	0.061	1.620 (1.235–2.125)	0.0004	2.580 (1.348–4.937)	0.004
Coexpression	7.037 (2.223–22.269)	0.001	1.650 (0.724–3.757)	0.233	30343 (1.791–6.242)	0.0001

and reviewed by two investigators (M.P. Humphries and A.M. Shaaban) and quantified (Leica Aperio positive pixel count algorithm, version 9).

Statistical analysis

ROC curves were generated to obtain relevant cutoffs (22). Associations with disease-free and overall survival (DFS, from initial diagnosis to the diagnosis of local or distant recurrence; OS, from initial diagnosis to death) were analyzed (Kaplan–Meier plots, log-rank test). HRs were determined by Cox regression. Follow-up patient information was updated in June 2013 and survival periods calculated. Patients were censored at the last day they were known to be alive. Variables were entered in univariate and multivariate analysis (Cox proportional hazards regression model). Gene expression *P* values were adjusted for multiple testing using the FDR method (Benjamini–Hochberg procedure).

Results

Gender comparison of gene expression

Hierarchical agglomerative clustering revealed differential gene expression patterns in MBC and FBC (Fig. 1A). Unsupervised clustering revealed three distinct gender-specific clusters. The top gene cluster displayed higher expression in MBC. The middle cluster showed lower expression in MBC, whereas the bottom cluster was overrepresented in MBC. Further analysis of the top cluster showed components of the translational initiation machinery were overexpressed in MBC compared with FBC, notably genes associated with translational initiation pathway. This was confirmed through mining an independent MBC dataset (Fig. 1B; ref. 10) and also by interrogation of Oncomine, which showed higher expression of *eIF4E* and *eIF5* in breast and lung cancer compared with matched normal tissue. When these biomarkers were compared for gender, eIF4E and eIF5 expression was proportionately higher in male breast but not lung cancer (Supplementary Fig. S2).

eIF4E and eIF5 expression are independently prognostic in MBC

Having identified gender-specific differences in eIF gene expression, we examined this immunohistochemically in 697 MBCs: training set (n = 477) and validation set (n = 220; ref. 9). Cytoplasmic expression was present in invasive tumor cells for all family members examined except eIF3, which was consistently negative, despite positive staining of colon-positive control tissue (Supplementary Fig. S3). Training and validation sets were scored semiquantitatively for each biomarker, taking account of intensity of staining and percentage of positive tumor cells. Representative staining for each eIF is shown in Supplementary Fig. S3. ROC curves were plotted and used to determine the optimum cut-off

Published OnlineFirst December 16, 2016; DOI: 10.1158/1078-0432.CCR-16-1952

value for each antibody. These were eIF1, 5.5; eIF2, 4.75; eIF4E, 5.77; and eIF5, 6.41 (Supplementary Fig. S3).

Kaplan–Meier survival curves showing the impact of eIF expression on OS and DFS are shown (Fig. 2). Expression of eIF4E and eIF5 was associated with worse OS. This relationship was also observed in the validation set and remained upon multivariate analysis in the larger training set when adjusted for age, tumor size, lymph node positivity, and grade (Table 2), even with disparity in significance of lymph node status between the two datasets; we attribute this to differences in the weighting of live/dead in each dataset. Alternatively, this may reflect the lack of complete data on lymph node status in both cohorts (Table 1); despite our best efforts, we were unable to obtain this. Significance remained when the training and validation sets were combined ($n = 697$ cases; Table 2).

As only eIF4E and eIF5 impacted on survival, we examined the effects of their coexpression. Low expression was determined for cases with scores below the defined cut-off point: <5.77 for eIF4E and <6.41 for eIF5 ($n = 96$). High expression: >5.77 for eIF4E and >6.41 for eIF5 ($n = 14$). Cases that overexpressed eIF4E and eIF5 (>5.77, >6.41, respectively) had significantly shorter survival compared with those who expressed eIF4E and eIF5 at lower levels (<5.77, <6.41, respectively; Fig. 3). Cases that were high for one of the proteins fell between both curves (data not shown). Coexpression of eIF4E and eIF5 remained significant upon multivariate analysis [$P = 0.001$; HR, 7.037 (2.223–22.2)] in the training set (Table 2). Correlations between eIF4E expression with PR ($P < 0.001$) and low tumor grade ($P < 0.036$) were observed, while AR correlated with eIF5 ($P < 0.035$), with a trend toward correlation with PR and low grade (Supplementary Table S1). No significant correlation with clinicopathologic parameters was observed in cases that coexpressed eIF4E and eIF5, although trends with lower grade and PR were suggested.

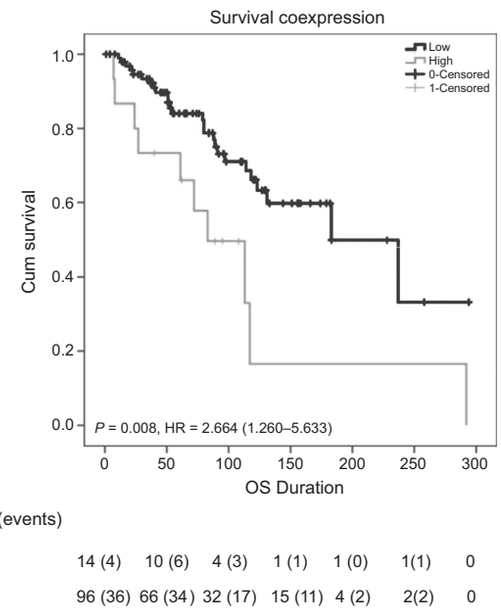

Figure 3.

Coexpression of eIF4E and eIF5 significantly impacts on MBC survival by Kaplan–Meier survival analysis. Cases that coexpressed eIF4E and eIF5 were stratified into low (score <5.77, <6.41, respectively; $n = 96$) or high (score >5.77, >6.41, respectively; $n = 14$) expression. Cases that overexpressed eIF4E and eIF5 had significantly shorter survival compared with those who expressed eIF4E and eIF5 at lower levels. Gray line, high expression; black line, lower expression, log-rank test.

BEZ235/everolimus combination therapy alters eIF4E and 5 expression

As overexpression of eIF4E and eIF5 was associated with reduced OS, we examined the effects of treatments known to impact on their signaling in a single MBC patient. In the pretreatment sample, strong cytoplasmic expression of eIF4E and eIF5 was observed (Fig. 4A and C, respectively). Strikingly in the posttreatment sample, a marked reduction in staining was observed for both biomarkers, 89% to 58% (eIF4E), 87% to 35% (eIF5), accompanied by a shift in location of eIF5 from the cytoplasm to the nucleus (Fig. 4B and D).

Discussion

To our knowledge, this is the largest study in MBC reported to date, examining more than 700 cases at the transcriptomic and immunohistochemical levels across four independent datasets. Key findings were upregulation of genes of the translational initiation pathway in MBC in two independent transcriptomic screens, followed by identification of eIF4E and eIF5 as independent predictors of survival, either when evaluated alone or when coexpressed, where there was an even stronger negative survival influence. We also provide evidence that the translational initiation pathway may be tractable by studying samples from an MBC patient who received an investigational combination of agents that target this pathway, namely BEZ235 and everolimus.

The role of initiation factors in the progression to a malignant phenotype is reported in many cancers, including, breast, head and neck, liver, prostate, bladder, gastric, colon, ovarian, glioma, lymphoma, non–small cell lung carcinoma, cervical, small intestine, and melanoma (20, 23–25). This has highlighted eIFs, notably eIF4E, as indicative of poor prognosis. Originally shown to be overexpressed in breast cancer (26), eIF4E is essential for translation and is a rate-limiting step in RNA recruitment to ribosomes (27). Indeed, most of the direct inhibitors of the eIF machinery are targeted toward eIF4E (28). Moreover, eIF4E and its associated binding proteins have been shown to correlate with survival duration in FBC, where cases with high expression of eIF4E relative to its binding proteins had significantly worse survival (20). Our results corroborate these and other findings where elevated eIF4E expression predicts poor survival in FBC (21, 29, 30).

Recently, 337 cases from our 477-case training set were examined independently, suggesting eIF4E expression had no prognostic effect in MBC (31). This anomaly might be explained by the different times used to estimate survival in the two studies. In this study, survival status was updated in June 2013 (by S. Sundara Rajan), while survival data in the cases used by Millican-Slater and colleagues (31) were earlier, 2008 to 2009, and only available for 187 cases. As well as using the most up to date survival information available, this emphasizes the need for inclusion of sufficiently large numbers of samples for robust validation studies when estimating the effects of biomarkers on survival, as widely discussed (32, 33). The large number of cases in our training ($n = 477$) and validation ($n = 220$) cohorts with follow-up on

Published OnlineFirst December 16, 2016; DOI: 10.1158/1078-0432.CCR-16-1952

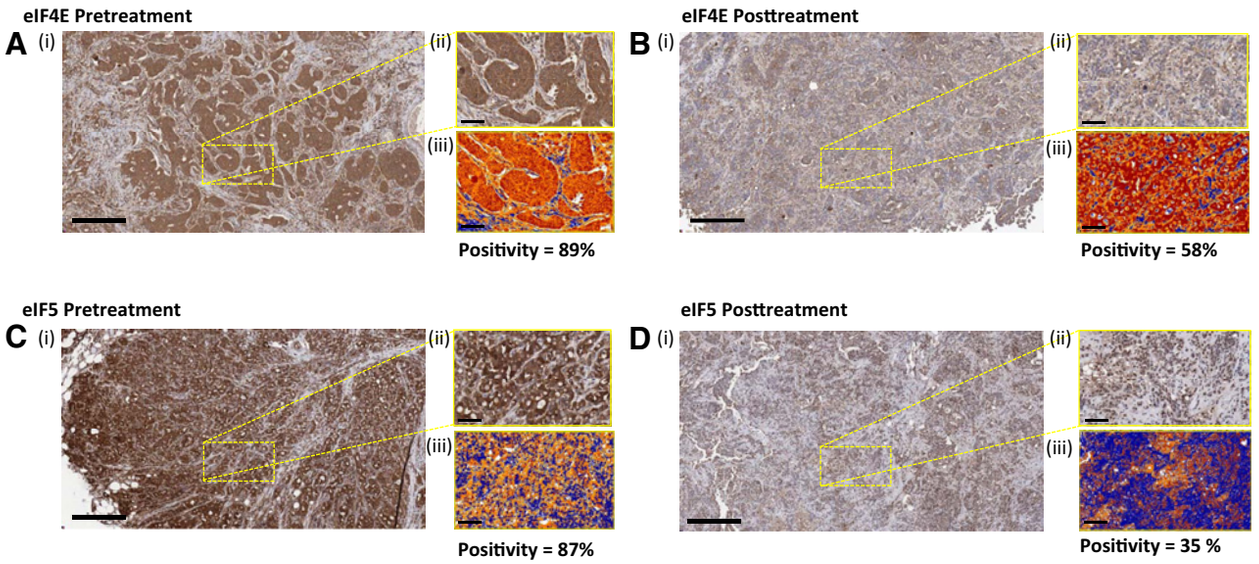

Figure 4.

BEZ235/everolimus combination therapy reduces eIF4E and eIF5 expression. **A–D,** (i) eIF4E and eIF5, expression in BEZ235/everolimus pre- and posttreatment patient samples, respectively; (ii) exploded views of a higher magnification of eIF4E and eIF5 staining in pre- and posttreatment patient samples, respectively; (iii) the positive pixel counting analysis images of the eIF4E and eIF5 higher magnification images from pre- and posttreatment patient samples, respectively. Scales bar (**A–D,** i), 300 μm; those on higher magnification and positive pixel analysis images = 60 μm.

>70% as well as concordance with previous literature (20, 21, 29, 30) are significant strengths, all pointing toward eIF4E being a poor prognostic factor in breast cancer, irrespective of gender. Given that we wished to identify potential gender-specific differences in gene expression in breast cancer, this result may be perceived as surprising. However, there are multiple examples of biomarkers being expressed in different, or even the same type, of breast cancer, but which are only of clinical use when expressed above a certain threshold (reviewed in ref. 34). Interestingly, a search on Oncomine showed that *eIF4E* and *eIF5* were not only increased in tumor versus normal breast and lung cancers, but that *eIF4E* and *eIF5* expression was proportionately higher in MBC when genders were compared, substantiating our findings. However, although we have shown *eIF4E* and *eIF5* are elevated in MBC, this does not preclude their expression and targeting in FBC. As we move toward personalized medicine, case-specific biomarker expression and their quantitative expression levels should help optimize tailored therapies for breast cancer in both genders.

As reported elsewhere (1, 35–37), our MBC cohort was almost universally ER^+, expressed in >90% of cases. As previous gene expression profiling studies indicate that MBC shares more features with ER^- FBC than ER^+ FBC (9), it is of interest to note that eIF4E overexpression has also been reported to negatively impact survival in triple-negative FBC (38). Thus, as well as sharing genomic similarities, this could indicate that ER^+ MBCs share a prognostic biomarker with ER^- FBC.

eIF5 is essential in the translation initiation process, responsible for the association of eIF2 with Met-tRNA (39), yet its precise role in cancer pathogenesis remains elusive. To our knowledge, this is the first time it has been shown to negatively affect survival duration in MBC. Interestingly, chromosome 3q26, the gene locus of *eIF5*, is amplified in breast cancer cell lines (40). Both eIF4E, eIF5, and combinations remained significant, remaining upon multivariate Cox regression analysis; however, this significance was reduced in our validation set, which we attribute to sample size, as follow-up length and treatment regimens were similar in both datasets (Table 1).

Despite detecting eIF3 mRNA in both MBC and FBC by qRT-PCR (data not shown), we were unable to detect protein expression by IHC. Expression in our positive control tissue eliminated the possibility of poor antibody efficacy or influence of other preanalytic factors. Nevertheless, there is immunohistochemical evidence that eIF3 expression is decreased in pancreatic cancer (24, 41). Further evidence from cancer profiling arrays shows general downregulation of *eIF3* in human tumors (24), which may explain its lack of expression.

The recognized contribution of eIFs to tumorigenesis has led to their investigation as therapeutically tractable targets, particularly using antisense approaches or small-molecule inhibitors (42). A phase I clinical trial showed reduction of eIF4E protein by up to 65% by an antisense oligonucleotide (LY2275796) in most of the 30 patients tested (43). Other targets of eIFs include PI3K and mTOR inhibitors. Rapamycin and analogues, upstream signaling inhibitors of translation initiation, are now in the clinic (44–46). We assessed eIF4E and eIF5 expression in an MBC patient who was treated with agents known to impact these signaling pathways, namely the mTOR inhibitor everolimus (Afinitor/RAD001) given in combination with BEZ235, an inhibitor of class I PI3K molecules and the mTORC1 and mTORC2 complexes. This clearly demonstrated a striking reduction in the expression of eIF4E and eIF5 (>50%) in the posttreatment samples. As the mTORC1/2 pathways are upstream of eIF4E (47), we predict their inhibition may result in declining levels of eIF proteins. Another study showed a reduction in eIF4E expression in approximately one third of breast cancers following treatment with everolimus (48). As overexpression of both eIF4E and eIF5 was associated with

Published OnlineFirst December 16, 2016; DOI: 10.1158/1078-0432.CCR-16-1952

worse OS in MBC, it is tempting to speculate that action of the BEZ235/everolimus combination could deregulate their molecular pathways, resulting in reduction in their expression, leading to survival benefit, as stable disease was maintained for 18 months after the BEZ235/everolimus switch. However, it is worth noting that the patient had already been heavily treated with other chemo and endocrine agents prior to this switch, which may have contributed to the reduction in eIF4E and eIF5 expression we report. Nevertheless, this intriguing result is supported by *in vivo* animal data in which suppressing mTOR activity and its downstream translational regulators delayed breast cancer progression (49). Clearly, further validation is required. Lack of specific male breast cancer cell line models, precludes this *in vitro*; potentially, this could be considered in the context of MBC-specific clinical trials, for example, as recommended by the International Male Breast Cancer Program (50). Another interesting observation was the relocation of eIF5 from a cytoplasmic to a nuclear location in the posttreatment sample. As the association of eIF2 with Met-tRNA by eIF5 occurs in the cytoplasm (39), the biological reasons for its presence in the nucleus are unknown.

In summary, gene expression analysis revealed that, compared with FBC, genes involved in the translational initiation pathway are overexpressed in MBC, corroborated by *in silico* validation in an independent dataset and immunohistochemical analysis demonstrating that overexpression of eIF4E and eIF5 are predictive of reduced patient survival in 697 MBCs with long-term follow-up. Together with our data on pre- and posttreatment evaluation of these biomarkers in an MBC patient, our findings suggest that MBCs that overexpress eIF4E and eIF5 might be considered as candidates for treatment with agents that target the translation machinery in cancer. Indeed preclinical data support the use of inhibition of translation initiation as an emerging new paradigm in cancer therapy (51).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Acknowledgments

The Breast Cancer Now Tissue Bank provided cases. Drs. Thomas Hughes, Rebecca Millican-Slater, and Prof. Andrew Hanby (University of Leeds and St James's University Hospital, Leeds, United Kingdom) gave helpful comments on manuscript drafts. Dr. Callari and the personnel of Tissue Bank of the Fondazione IRCCS Istituto Nazionale dei Tumori Milan helped in mining his previously published MBC dataset (10) and sample collection, respectively. Special thanks to Dr. David Cairns, Prof. Charles Taylor, and Alex Wright, University of Leeds, for advice on statistical analysis and positive pixel algorithms, respectively.

Grant Support

This study was funded by Yorkshire Cancer Research (grant L278). Breast Cancer Now (formerly Breast Cancer Campaign, grant 2007MayPR02) provided funding for the accrual and construction of the MBC TMAs. The Breast Cancer Research Trust contributed toward costs of genomic analysis. This work was partially supported by grants from the Italian Association for Cancer Research and the Swedish Cancer Society.

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Received August 3, 2016; revised October 26, 2016; accepted November 19, 2016; published OnlineFirst December 16, 2016.

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A Case-Matched Gender Comparison Transcriptomic Screen Identifies eIF4E and eIF5 as Potential Prognostic Markers in Male Breast Cancer

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Clin Cancer Res 2017;23:2575-2583. Published OnlineFirst December 16, 2016.