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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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Conflicts of interest

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Running head: Prognostic biomarkers in male breast cancer

Abstract

Purpose: Breast cancer (BC) affects both genders, but is understudied in men. Although still rare, male BC 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 BC 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 post-treatment samples from a male BC patient receiving Everolimus and PI3K/mTOR inhibitor.

Results: Gender-specific gene expression patterns were identified. eIF transcripts were up-regulated 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 co-expressed (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); co-expression 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 male BC patients overexpressing eIF4E and eIF5. With mTOR inhibitors which target this pathway now in the clinic, these biomarkers may represent new targets for therapeutic intervention, although further independent validation is required.

Keywords: breast cancer; genomics; eIF; survival; mTOR inhibitor

Statement of significance

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 co-expressed. Samples from a patient receiving a combination of agents targeting this pathway, suggests 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 immunohistochemistry [1-5], genetics [6-11] and more recently, epigenetics [12-15]. Of note, whilst 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 recognised in FBC where molecular profiling has identified different subgroups which 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 remodelling, metabolism and protein synthesis via genes involved in translational initiation, including eIF4E [10] which are often upregulated in MBC compared to FBC. Further work has identified 2 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 *et al* documented differential driver genes in MBC vs FBC [16]. Most recently a distinct repertoire of genetic alterations were 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 post-progression sample, with little change at the genomic level, whilst 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 if differences in specific biomarkers affected clinical outcome in men using a training set of 477 and a validation set of 220 cases. Finally we analysed expression of these biomarkers in pre- and post-treatment samples from a MBC patient who received a combination of the PI3K/mTOR inhibitors BEZ235 and Everolimus [17].

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 (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 (TMAs; n= 446, constructed as described [1]) and 31 full faced sections, plus a validation set (220 cases on TMAs [9]), was used in immunohistochemistry. Patient characteristics are shown in Table 1. Details on the datasets used in the explorative and validation phases is provided (Figure S1). Cases were pseudo-anonymised and data analysed anonymously.

Gene expression

Five x 10µm sections applied to Almac Diagnostics (Craigavon, UK) 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 normalised and transformed data with FDR threshold of 5% and a fold-change significance of 1%. Representative heat maps 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.

Immunohistochemistry

REMARK criteria were employed [19]. Immunohistochemistry was conducted as previously described, using well validated antibodies [20], including: eIF1 (Abcam - ab118979. 1:200); eIF2 (Abcam - ab32157. 1:150); eIF3 (Abcam - ab171419. 1:150); eIF4E (Santa Cruz - sc-9976. 1:400); eIF5 (Abcam - ab32443. 1:300). Cases were batch stained for each antibody with recommended controls. TMAs were digitised (x40, Leica-Aperio AT2 ScanScope scanner; Leica Biosystems, UK). Each TMA core was viewed using in-house software and assessed semi-quantitatively for each biomarker taking account of staining intensity and percentage of tumour cells. Overall scores were averaged from either duplicate or triplicate cores which 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 co-scoring of 10% (CABS, trainee histopathologist), overseen by AMS, specialised breast consultant histopathologist. Where disagreement was reported (score >2; n=5) cases were re-reviewed to reach consensus. Excellent strength of agreement was observed between scorers using Inter-Class Correlation Coefficients (eIF1 0.911 (95%CI 0.769-0.944), eIF2 0.846 (95%CI 0.736-0.910), eIF4E 0.882 (95%CI 0.755-0.913), eIF5 0.865 (95%CI 0.769-0.922). Scores were indeterminable in 49 cases due to core loss/exhaustion during processing, well-recognised with TMAs.

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

Pre- and post-treatment biopsies were obtained from a 66 year old Caucasian male diagnosed in 2006 with ER+, 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 (pre-treatment sample). Standard adjuvant chemotherapy commenced, with 5 weeks of 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 stabilisation he received fulvestrant. After 8 months, node progression was noted and the patient was switched to BEZ235 (200mg orally, twice daily) plus sub-therapeutic Everolimus (2.5mg, 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 (post-treatment sample). eIF4E and eIF5 expression was assessed immunohistochemically in the pre- and post-treatment samples, as described above and reviewed by two investigators (MPH and AMS) and quantified (Leica Aperio positive pixel count algorithm, version 9).

Statistical analysis

Receiver operator curves were generated to obtain relevant cut-offs [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 analysed (Kaplan–Meier plots, log rank test). Hazard ratios 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 false discovery rate method (Benjamini-Hochberg procedure).

Results

Gender comparison of gene expression

Hierarchical agglomerative clustering revealed differential gene expression patterns in MBC and FBC (Figure 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 while the bottom cluster was over represented 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 [10] (Figure 1B) and also by interrogation of Oncomine™ which showed higher expression of eIF4E and eIF5 in breast and lung cancer compared to matched normal tissue. When these biomarkers were compared for gender, eIF4E and eIF5 expression was proportionately higher in male breast but not lung cancer (Figure 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), validation set (n=220) [9]. Cytoplasmic expression was present in invasive tumour cells for all family

members examined except eIF3, which was consistently negative, despite positive staining of colon positive control tissue (Figure S3). Training and validation sets were scored semi-quantitatively for each biomarker, taking account of intensity of staining and percentage of positive tumour cells. Representative staining for each eIF is shown in Figure S3. R.O.C curves were plotted and used to determine the optimum cut-off value for each antibody. These were: eIF1, 5.5; eIF2, 4.75; eIF4E, 5.77; and eIF5, 6.41 (Figure S3).

Kaplan Meier survival curves showing the impact of eIF expression on OS and DFS are shown (Figure 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, tumour size, lymph node positivity and grade (Table 2), even with disparity in significance of lymph node status between the 2 data sets; 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 co-expression. Low expression was determined for cases with scores below the defined cut 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 over-expressed eIF4E and eIF5 (>5.77, >6.41 respectively) had significantly shorter survival compared to those who expressed eIF4E and eIF5 at lower levels (<5.77, <6.41 respectively; Figure 3).

Cases which were high for one of the proteins fell between both curves (data not shown). Co-expression 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 tumour grade (P<0.036) were observed, while AR correlated with eIF5 (P<0.035), with a trend towards correlation with PR and low grade (Table S1). No significant correlation with clinico-pathological parameters was observed in cases which co-expressed eIF4E and eIF5, although trends with lower grade and PR were suggested.

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 signalling in a single MBC patient. In the pre-treatment sample, strong cytoplasmic expression of eIF4E and eIF5 was observed (Figure 4A, C, respectively). Strikingly in the post-treatment 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 (Figure 4B, D).

Discussion

To our knowledge this is the largest study in MBC reported to date, examining over 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 co-expressed, 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 a MBC patient who received an investigational combination of agents which 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 (NSCLC), 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 [29-31].

Recently, 337 cases from our 477-case training set were examined independently, suggesting eIF4E expression had no prognostic effect in MBC [32]. This anomaly might be explained by the different times used to estimate survival in the 2 studies. In this study survival status was updated in June 2013 (by SSR) while survival data in the cases used by Millican-Slater et al [32] was earlier, 2008-2009, and only

available for 187 cases. As well as using the most up to date survival information available, this emphasises 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 [33, 34]. The large number of cases in our training (n=477) and validation (n=220) cohorts with follow up on >70% as well as concordance with previous literature [20, 29-31] are significant strengths, all pointing towards 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 [35]). Interestingly, searching of Oncomine™ showed that eIF4E and eIF5 were not only increased in tumour 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, while we have shown eIF4E and eIF5 are elevated in MBC, this does not preclude their expression and targeting in FBC. As we move towards personalised medicine, case-specific biomarker expression and their quantitative expression levels should help optimise tailored therapies for breast cancer in both genders.

As reported elsewhere [36-38, 1], 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 [39]. 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 [40] 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 [41]. 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 immunohistochemistry. Expression in our positive control tissue eliminated the possibility of poor antibody efficacy or influence of other pre-analytical factors. Nevertheless, there is immunohistochemical evidence that eIF3 expression is decreased in pancreatic cancer [42, 24]. Further evidence from cancer profiling arrays shows general downregulation of eIF3 in human tumours [24], which may explain its lack of expression.

The recognised contribution of eIFs to tumorigenesis has led to their investigation as therapeutically tractable targets, particularly using antisense approaches or small molecule inhibitors [43]. A phase one clinical trial showed reduction of eIF4E protein

by up to 65% by an antisense oligonucleotide (LY2275796) in most of the 30 patients tested [44]. Other targets of eIFs include PI3K and mTOR inhibitors. Rapamycin and analogues, upstream signalling inhibitors of translation initiation, are now in the clinic [45-47]. We assessed eIF4E and eIF5 expression in a MBC patient who was treated with agents known to impact these signalling 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 expression of eIF4E and eIF5 (>50%) in the post-treatment samples. As the mTORC1/2 pathways are upstream of eIF4E [48], 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 [49]. As over-expression of both eIF4E and eIF5 was associated with worse overall survival 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 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 [50]. 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 e.g. as recommended by the International Male Breast Cancer Program [51]. Another interesting observation was the relocation of eIF5 from a

cytoplasmic to a nuclear location in the post-treatment sample. As the association of eIF2 with Met-tRNA by eIF5 occurs in the cytoplasm [40], the biological reasons for its presence in the nucleus is unknown.

In summary, gene expression analysis revealed that, compared to FBC, genes involved in the translational initiation pathway are over-expressed in MBC, corroborated by *in silico* validation in an independent data set and immunohistochemical analysis demonstrating that over-expression 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 post-treatment evaluation of these biomarkers in a MBC patient, our findings suggest that MBCs that overexpress eIF4E and eIF5 might be considered as candidates for treatment with agents which target the translation machinery in cancer. Indeed pre-clinical data support the use of inhibition of translation initiation as an emerging new paradigm in cancer therapy [52].

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Table 1

Clinicopathological 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)*	18 (8)*
-	291 (65	157 (71)
Unknown	149 (34)	45 (20)

*Confirmed by FISH/CISH

Table 2

Univariate and multivariate analysis of eIF4E and eIF5 expression in MBC

Univariate analysis (all biomarkers)						
Variable	**Training set**		**Validation set**		**Combined dataset**	
	Hazard ratio (CI)	P-value	Hazard ratio (CI)	P-value	Hazard ratio (CI)	P-value
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
Co-expression	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						
	Hazard ratio (CI)	P-value	Hazard ratio (CI)	P-value	Hazard ratio (CI)	P-value
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)						
Variable	**Training set**		**Validation set**		**Combined dataset**	
	Hazard ratio (CI)	P-value	Hazard ratio (CI)	P-value	Hazard ratio (CI)	P-value

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 (co-expression of EIF4E and EIF5)						
Variable	**Training set**		**Validation set**		**Combined dataset**	
	Hazard ratio (CI)	P-value	Hazard ratio (CI)	P-value	Hazard ratio (CI)	P-value
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
Co-expression	7.037 (2.223-22.269)	0.001	1.650 (0.724-3.757)	0.233	30343 (1.791-6.242)	0.0001

Figure legends

Figure 1

Identification of eIF pathway up regulation 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 over-expressed 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 = over-expression; red = under-expression.

Figure 2

The effect of eIF expression on disease-free and overall survival in MBC by Kaplan-Meier survival analysis

Effects on OS are shown in A, C, E, G and DFS in B, D, F, H. A, B = eIF1; C, D; eIF2; E, F = eIF4E; G, H = eIF5. Black line = high expression, Grey line = low expression, dichotomised by R.O.C. analysis and analysed by log rank test.

Figure 3

Co-expression of eIF4E and eIF5 significantly impacts on MBC survival by Kaplan-Meier survival analysis

Cases which co-expressed 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

over-expressed eIF4E and eIF5 had significantly shorter survival compared to those who expressed eIF4E and eIF5 at lower levels. Black line = high expression, Grey line = lower expression, log rank test.

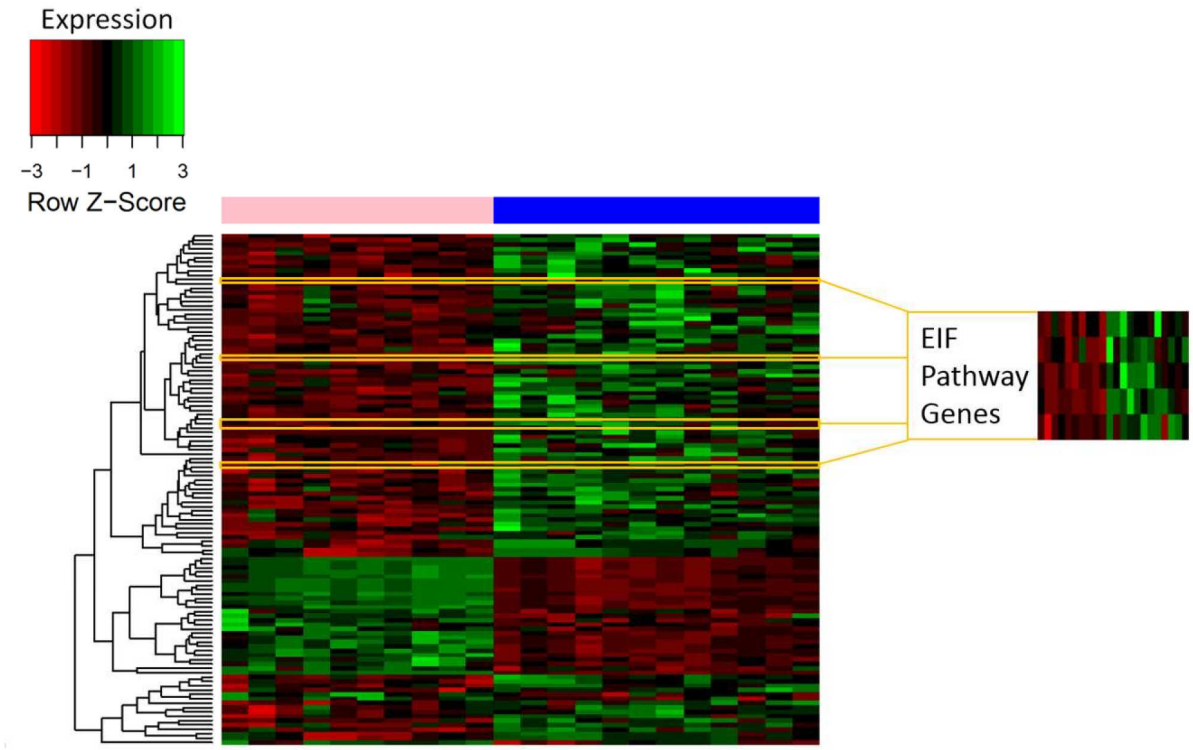
Figure 4

BEZ235/Everolimus combination therapy reduces eIF4E and eIF5 expression

A – D i) display eIF4E and eIF5, expression in BEZ235/Everolimus pre- and post-treatment patient samples, respectively. A – D ii) show exploded views of a higher magnification of eIF4E and eIF5 staining in pre- and post-treatment patient samples respectively. A – D iii) display the positive pixel counting analysis images of the eIF4E and eIF5 higher magnification images from for pre- and post-treatment patient samples respectively. Scales on images A-D i) =300 μm, those on higher magnification and positive pixel analysis images = 60 μm.

Figure 1

1A



1B

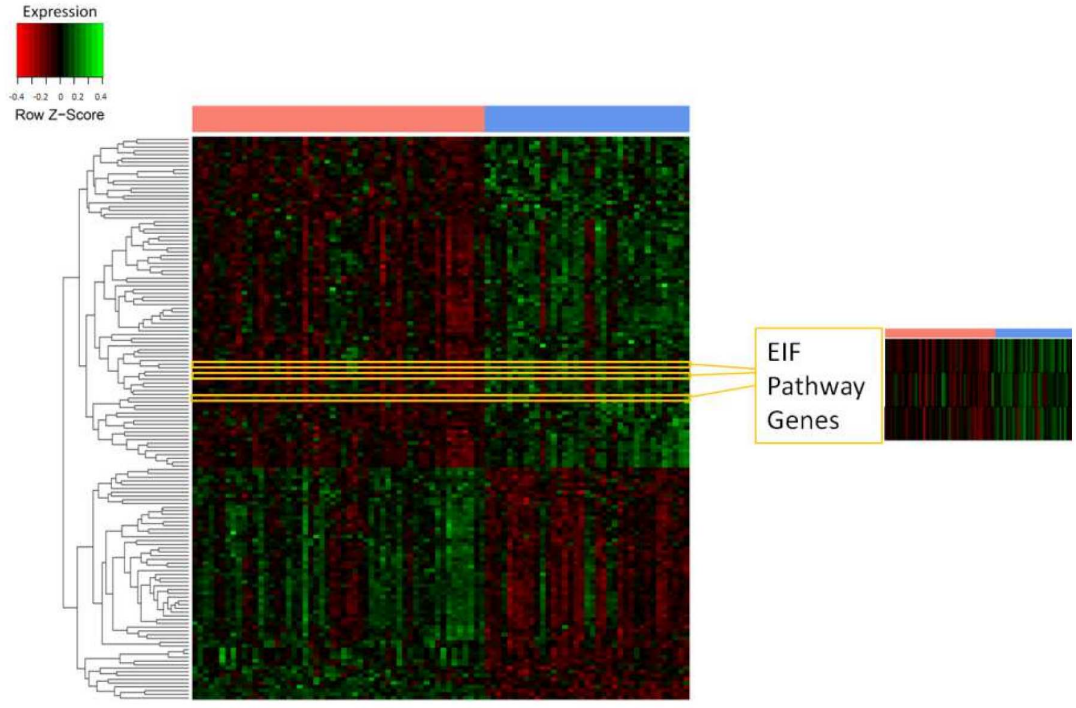


Figure 2

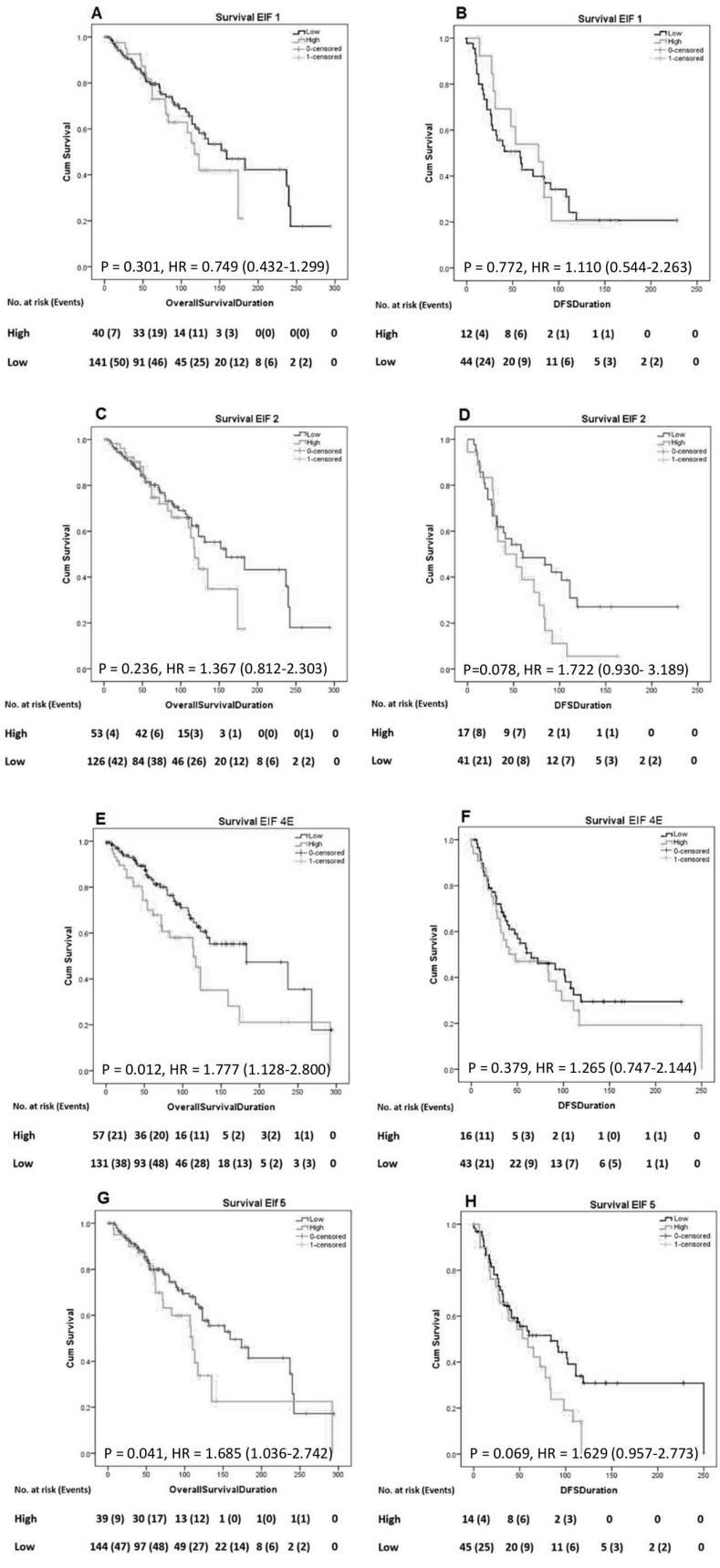
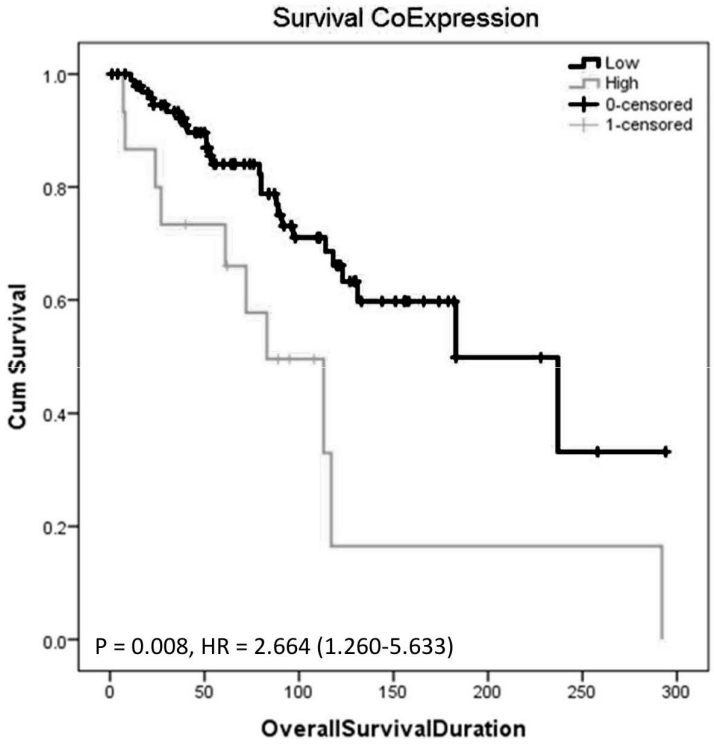

A
Survival EIF 1
Low
High
0-censored
1-censored
Cum Survival
P = 0.301, HR = 0.749 (0.432-1.299)
OverallSurvivalDuration
No. at risk (Events)
High 40 (7) 33 (19) 14 (11) 3 (3) 0(0) 0(0) 0
Low 141 (50) 91 (46) 45 (25) 20 (12) 8 (6) 2 (2) 0
B
Survival EIF 1
P = 0.772, HR = 1.110 (0.544-2.263)
DFSDuration
No. at risk (Events)
High 12 (4) 8 (6) 2 (1) 1 (1) 0 0
Low 44 (24) 20 (9) 11 (6) 5 (3) 2 (2) 0
C
Survival EIF 2
P = 0.236, HR = 1.367 (0.812-2.303)
OverallSurvivalDuration
No. at risk (Events)
High 53 (4) 42 (6) 15(3) 3 (1) 0(0) 0(1) 0
Low 126 (42) 84 (38) 46 (26) 20 (12) 8 (6) 2 (2) 0
D
Survival EIF 2
P=0.078, HR = 1.722 (0.930- 3.189)
DFSDuration
No. at risk (Events)
High 17 (8) 9 (7) 2 (1) 1 (1) 0 0
Low 41 (21) 20 (8) 12 (7) 5 (3) 2 (2) 0
E
Survival EIF 4E
P = 0.012, HR = 1.777 (1.128-2.800)
OverallSurvivalDuration
No. at risk (Events)
High 57 (21) 36 (20) 16 (11) 5 (2) 3(2) 1(1) 0
Low 131 (38) 93 (48) 46 (28) 18 (13) 5 (2) 3 (3) 0
F
Survival EIF 4E
P = 0.379, HR = 1.265 (0.747-2.144)
DFSDuration
No. at risk (Events)
High 16 (11) 5 (3) 2 (1) 1 (0) 1 (1) 0
Low 43 (21) 22 (9) 13 (7) 6 (5) 1 (1) 0
G
Survival Elf 5
P = 0.041, HR = 1.685 (1.036-2.742)
OverallSurvivalDuration
No. at risk (Events)
High 39 (9) 30 (17) 13 (12) 1 (0) 1(0) 1(1) 0
Low 144 (47) 97 (48) 49 (27) 22 (14) 8 (6) 2 (2) 0
H
Survival EIF 5
P = 0.069, HR = 1.629 (0.957-2.773)
DFSDuration
No. at risk (Events)
High 14 (4) 8 (6) 2 (3) 0 0 0
Low 45 (25) 20 (9) 11 (6) 5 (3) 2 (2) 0

Figure 3

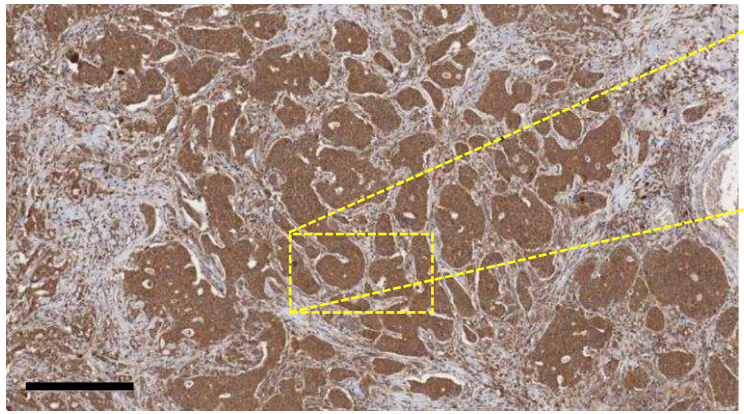


No. at risk (Events)							
High	**14 (4)**	**10 (6)**	**4 (3)**	**1 (1)**	**1(0)**	**1(1)**	**0**
Low	**96 (36)**	**66 (34)**	**32 (17)**	**15 (11)**	**4 (2)**	**2 (2)**	**0**

Figure 4

eIF4E Pre-treatment

A i)



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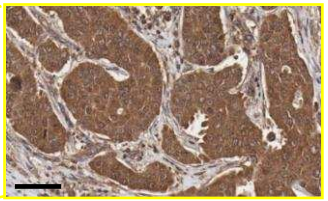
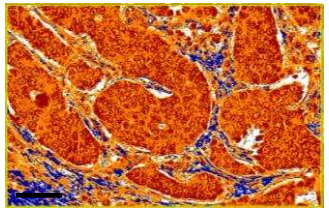


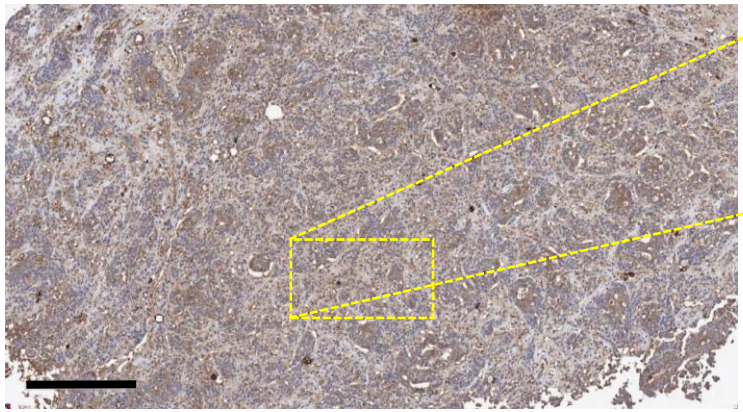
iii)



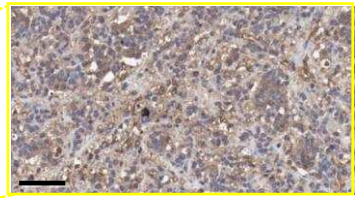
Positivity = 89%

eIF4E Post-treatment

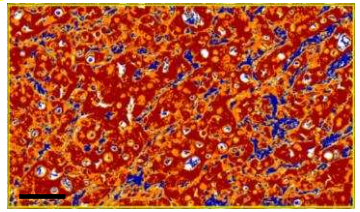
B i)



ii)



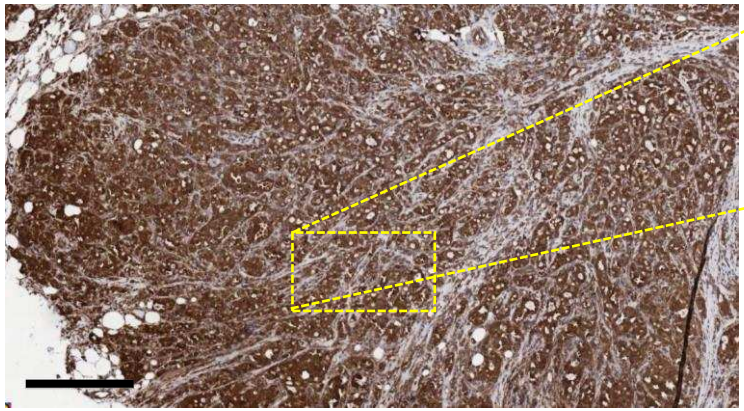
iii)



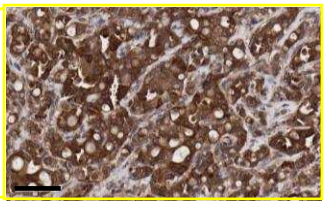
Positivity = 58%

eIF5 Pre-treatment

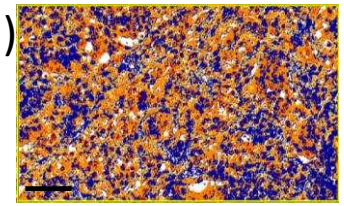
C i)



ii)



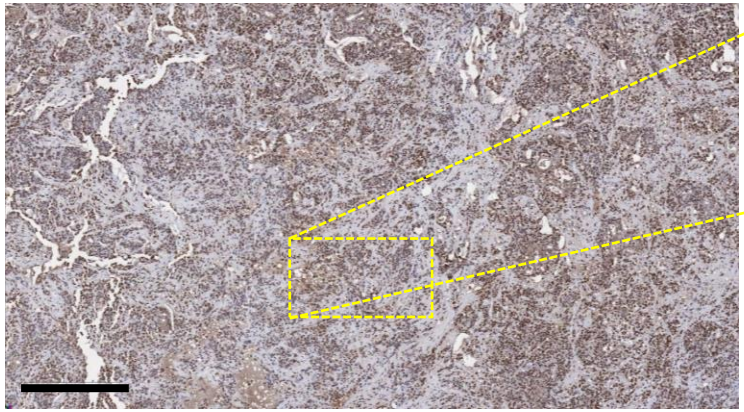
iii)



Positivity = 87%

eIF5 Post-treatment

D i)



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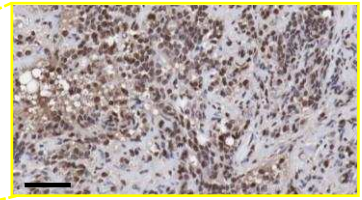

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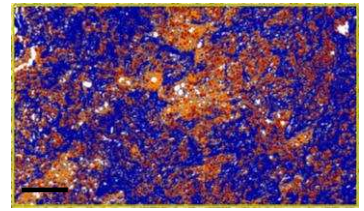

Positivity = 35 %