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MYC functions are specific in biological subtypes of breast cancer and confers resistance to endocrine therapy in luminal tumours

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Background: *MYC* is amplified in approximately 15% of breast cancers (BCs) and is associated with poor outcome. c-MYC protein is multi-faceted and participates in many aspects of cellular function and is linked with therapeutic response in BCs. We hypothesised that the functional role of c-MYC differs between molecular subtypes of BCs.

Methods: We therefore investigated the correlation between c-MYC protein expression and other proteins involved in different cellular functions together with clinicopathological parameters, patients' outcome and treatments in a large early-stage molecularly characterised series of primary invasive BCs ($n=1106$) using immunuohistochemistry. The METABRIC BC cohort ($n=1980$) was evaluated for *MYC* mRNA expression and a systems biology approach utilised to identify genes associated with *MYC* in the different BC molecular subtypes.

Results: High *MYC* and c-MYC expression was significantly associated with poor prognostic factors, including grade and basal-like BCs. In luminal A tumours, c-MYC was associated with ATM ($P=0.005$), Cyclin B1 ($P=0.002$), PIK3CA ($P=0.009$) and Ki67 ($P<0.001$). In contrast, in basal-like tumours, c-MYC showed positive association with Cyclin E ($P=0.003$) and p16 ($P=0.042$) expression only. c-MYC was an independent predictor of a shorter distant metastases-free survival in luminal A LN+ tumours treated with endocrine therapy (ET; $P=0.013$). In luminal tumours treated with ET, *MYC* mRNA expression was associated with BC-specific survival ($P=0.001$). In ER-positive tumours, *MYC* was associated with expression of translational genes while in ER-negative tumours it was associated with upregulation of glucose metabolism genes.

Conclusions: c-MYC function is associated with specific molecular subtypes of BCs and its overexpression confers resistance to ET. The diverse mechanisms of c-MYC function in the different molecular classes of BCs warrants further investigation particularly as potential therapeutic targets.

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In a meta-analysis of 3797 patients, V-Myc Avian Myelocytomatosis Viral Oncogene Homolog (*MYC*) is amplified in 15.7% of breast cancers (BCs) and is associated with higher tumour grade, advanced stage and progesterone receptor (PgR)-negative tumours resulting in an association with poor overall survival and disease recurrence independent of existing prognostic factors (Deming *et al*, 2000). The c-MYC protein is multi-faceted and participates in many aspects of cellular function, including replication, growth, metabolism, differentiation and apoptosis (Liao and Dickson, 2000). The mechanisms of c-MYC action in controlling these different roles remain poorly understood. However, it may be context or isoform dependent whether c-MYC promotes cell proliferation or apoptosis (Deming *et al*, 2000; Liao and Dickson, 2000; Patel *et al*, 2004).

The expression of c-MYC is regulated by oestrogen via the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI-3 kinase) pathways, among other signalling pathways, promoting cell proliferation and survival (Liao *et al*, 2000). Antioestrogen treatment, including aromatase inhibitors, tamoxifen and faslodex, all downregulate *MYC* mRNA inducing cell cycle arrest (Carroll *et al*, 2002). There is also strong evidence that c-MYC has a role in the development of antioestrogen resistance where it is frequently overexpressed during progression and distant relapse of oestrogen receptor (ER)-positive BCs treated with adjuvant hormonal therapy (Planas-Silva *et al*, 2007). Clinical evidence also suggests that c-MYC and B-cell CLL/lymphoma 2 (Bcl-2) act in concert to promote lymph node metastasis in early BCs (Sierra *et al*, 1999). c-MYC overexpression can also cause DNA damage and subsequently trigger apoptosis via DNA damage response pathways. ATM serine/threonine kinase (ATM), a critical DNA double-strand break repair protein, is upregulated in response to such DNA damage and is necessary for p53 activation and suppression of tumour development.

In a recent study, it was demonstrated that c-MYC expression was upregulated through the cross-talk between ER and human epidermal growth factor receptor 2 (HER2) in BC cells (Chen *et al*, 2015). Cross-talk between ER and HER2 regulates c-MYC-mediated glutamine metabolism in aromatase inhibitor-resistant BC cells.

Therapeutic strategies aimed at targeting c-MYC have emerged, including interfering with c-MYC synthesis, dimerisation, stability and transcriptional activity (Vita and Henriksson, 2006; Albihn *et al*, 2010; Boddupally *et al*, 2012; Dang, 2012). However, the effectiveness of targeting c-MYC using single agents and failure to achieve long-lasting efficacy is observed because MYC function controls the expression of multiple genes and cancer cells can recover from oncogene addiction.

Thus targets aimed at inhibiting downstream signalling pathways in c-MYC-dependent tumours such as stress-response, metabolism and cell cycle pathways are currently the focus for new therapeutic opportunities (Dang, 2012). Several clinical trials focussed on these biological processes are currently underway (Horiuchi *et al*, 2014).

In this study, we hypothesised that the functional role of c-MYC differs between molecular subtypes of BCs and would influence the response for different type of adjuvant therapeutic regimens. We therefore investigated the correlation between *MYC* mRNA and c-MYC protein expression and other proteins involved in cell proliferation, DNA damage and apoptosis, in addition to clinicopathological parameters, outcome and treatments in a large cohort of molecularly characterised early-stage invasive primary breast carcinoma.

MATERIALS AND METHODS

***MYC* gene expression.** *MYC* gene expression was evaluated in a cohort of 1980 BC samples using the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) cohort (Curtis *et al*, 2012). RNA from fresh frozen tumours were subject to transcriptional profiling using the Illumina HT-12 v3 platform (San Diego, CA, USA), and the data were preprocessed and normalised as described previously (Curtis *et al*, 2012). In this cohort, patients with ER-positive and/or lymph node-negative tumours did not receive adjuvant chemotherapy, whereas those with ER-negative and/or lymph node-positive tumours received adjuvant chemotherapy.

c-MYC protein expression

Study patients. Immunohistochemistry was conducted using a large cohort of patients comprising a well-characterised consecutive series of early stage (TNM Stages I–III excluding T3 and T4 tumours) sporadic primary operable invasive BCs from patients (age $\leqslant$70 years) enrolled into the Nottingham Tenovus Primary Breast Carcinoma Series who presented at the Nottingham City Hospital between 1989 and 1998 ($n=1106$) and managed in accordance to a uniform protocol. Patients' clinical history, tumour characteristics, information on therapy and outcomes are prospectively maintained. Outcome data were collected on a prospective basis and included development and time to distant metastasis (DM) and BC-specific survival (BCSS). DM-free survival is defined as the time (in months) from the date of primary surgery to the appearance of DM. The BCSS is defined as the time (in months) from the date of primary surgery to the date of BC-related death. Luminal tumours were defined as ER+; triple-negative status were all those tumours not expressing ER, PgR and HER2; and HER2+ status was defined as all tumours overexpressing HER2 irrespective of ER status. Patients with luminal tumours had the best BCSS followed by those with triple-negative tumours (Supplementary Figure S1). Patients with HER2+ tumours had the poorest outcome.

Validating antibody specificity. The specificity of the anti-c-MYC primary antibody (Clone 9E100; Abcam Ltd, Cambridge, UK) was validated using western blotting (WB). WB was performed on whole-cell lysates of MDA-MB-468 human BC cell line (obtained from the American Type Culture Collection; Rockville, MD, USA) using 1 : 500 dilution of the primary antibody dilution, and 1 : 15000 horseradish peroxidase-conjugated secondary antibody (SA) (Li-cor Biosciences, Cambridge, UK). 5% milk (Marvel Original Dried Skimmed Milk, Premier Food Groups Ltd, St Albans, UK) was used for blocking. HRP-conjugated Anti-Rabbit β-actin (Clone AC-15; Sigma, Gillingham, UK) at 1 : 5000 was used as a house-keeping protein. A protein ladder (PageRuler Plus Prestained Protein Ladder, ThermoScientific, Waltham, MA, USA) was included. Chemiluminescence was used to visualise bands using Odyssey Fc (Li-cor Biosciences, Lincoln, NE, USA), which showed a single specific band at the right size (41 KDa) for c-MYC protein, confirming the specificity of the antibody (Supplementary Figure S2A).

Tissue arrays and immunohistochemistry. Tumour samples were arrayed as previously described (Abd El-Rehim *et al*, 2005). In brief, tissue cores with a diameter of 0.6 mm were punched from the representative tumour regions of each donor block. Cores were precisely arrayed into a new recipient paraffin blocks using a tissue microarrayer (Beecher Instruments, Sun Prairie, WI, USA). Immunohistochemical staining was performed on 4-μm thick sections using Novolink polymer detection system (Leica Biosystems, Newcastle, UK, RE7150-K), composed of Peroxidase Block, Post Primary Block, Novolink Polymer, 3,3′-diaminobenzidine (DAB) chromogen and substrate buffer and Novolink haematoxylin. Briefly, tissue slides were deparaffinised with xylene and rehydrated through three changes of alcohol. Heat-induced antigen epitope retrieval was performed in citrate buffer (pH 6.0) for 20 min using a microwave oven. Endogenous peroxidase activity

was blocked by Peroxidase Block for 5 min. Slides were washed with Tris-buffered saline (TBS, pH 7.6), followed by application of Protein Block for 5 min. Following another TBS wash, mouse monoclonal primary antibody for c-MYC (Clone 9E100; Abcam Ltd) at 1 : 100 in Leica antibody diluent (RE7133) was applied and incubated for 45 min. Slides were washed with TBS followed by incubation with Post Primary Block for 30 min followed by a TBS wash. Novolink polymer was applied for 30 min. DAB working solution made up of 1 : 20 DAB chromogen in DAB substrate buffer was prepared and applied for 5 min. Slides were counter-stained with Novolink haematoxylin for 6 min, dehydrated and coverslipped. Negative (omission of the primary antibody) and positive controls were included according to the manufacturer's datasheet of each antibody. Immunhistochemical staining and dichotomisation of the other biomarkers included in this study were as per previous publications (Rakha *et al*, 2007; Elsheikh *et al*, 2008; Rakha *et al*, 2009; Aleskandarany *et al*, 2010a,b, 2011, 2012; Habashy *et al*, 2013; Barros *et al*, 2014; Supplementary Table S1).

Evaluation of immunohistochemical staining. Assessment of staining was estimated subjectively on intensity corresponding to negative, weak, moderate and strong nuclear and/or cytoplasmic staining. Dichotomisation of c-MYC protein expression was based on the mean resulting in negative/weak (MYC negative) and moderate/strong (MYC positive) groups, which were selected prior to analysis.

Artificial neural network (ANN) model. A nonlinear ANN modelling-based approach was utilised to identify those gene probes associated with high *MYC* expression in ER-positive and -negative disease using the METABRIC cohort. A total of 48 803 probes were screened for each sample. The data-mining algorithm comprised a three layer multilayer perception architecture modified with a feed forward back-propagation algorithm and a sigmoidal transfer function, as previously described (Lancashire *et al*, 2010; Abdel-Fatah *et al*, 2014). The network momentum and learning rate were, respectively, set as 0.1 and 0.5. A parsimonious structure using two hidden nodes and three split Monte Carlo Cross validation were utilised to prevent over fitting. The output node was coded as 0 if a case was low *MYC* expression (median) and as 1 if it was above the median expression value. Inputs were ranked in ascending order based on their average classification error for the test subset. The top 200 predictive genes identified were then applied to an ANN-based network inference algorithm as described in earlier studies. This model predicted a weighted link (direction and magnitude) between each of the top 200 gene probe markers associated with *MYC* expression and every other marker in the top 200. The 100 strongest interactions based on the magnitude were then visualised as a map with Cyto-scape (Smoot *et al*, 2011).

External validation cohorts. For external validation of *MYC* mRNA expression, bc-GenExMiner v3.0 (Breast Cancer Gene-Expression Miner v3.2) online data set (http://bcgenex.centregau-ducheau.fr) was used. In this study, the gene correlation-targeted analysis offering the possibility to evaluate the correlation between MYC and candidate genes in ER+ and ER− BCs was used (Jezequel *et al*, 2012).

Statistical analysis. Statistical analysis was performed using the SPSS 21.0 statistical software (SPSS Inc., Chicago, IL, USA). Univariate and multivariate analyses were performed by chi-squared test, Log rank and Cox regression analysis, respectively. Survival curves were analysed by the method of Kaplan–Meier. A *P*-value <0.01 was considered significant. This study complied with reporting recommendations for tumour marker prognostic studies criteria (McShane *et al*, 2005). Recursive partitioning (Hothorn *et al*, 2006) was used to identify a cutoff in gene expression values such that the resulting subgroups have significantly different survival courses.

Ethics. This study was approved by the Nottingham Research Ethics Committee 2 under the title 'Development of a molecular genetic classification of breast cancer'.

RESULTS

MYC expression and clinicopathological parameters. High *MYC* mRNA expression was observed in 260 out of 1977 (13.1%) tumours. Positive cytoplasmic and nuclear expression of c-MYC protein was observed in 559 out of 1106 (50.5%) of invasive BC cases while 547 (49.5%) were negative Supplementary Figures S2B and C.

There was significant association between *MYC* mRNA and c-MYC protein expression with poor prognostic indicators of BCs. High *MYC* mRNA expression was associated with high grade ($P<0.001$) and Nottingham Prognostic Index ($P=0.001$) but not size or lymph node stage (Table 1). Positive c-MYC protein expression was significantly associated with higher tumour grade ($P=0.043$), mitotic frequency scores ($P=0.004$), nuclear pleomporphism ($P=0.012$) and lymph node stage ($P=0.046$, Table 1). c-MYC was also significantly associated with histological tumour type where more expression was observed in medullary-like tumours (22 out of 31, 71.0%) but was less likely to be expressed in lobular tumours (41 out of 106, 38.7%). However, there was no association between c-MYC and patient age, tumour size, tubule formation or lymphovascular invasion.

MYC expression and biomarkers. High *MYC* mRNA expression was significantly associated with ER and PgR-negative tumours (both $P<0.001$), HER2-positive ($P=0.001$) as well as triple-negative tumours ($P<0.001$, Table 1). There was no association between c-MYC protein expression with ER, PgR nor HER2 (Table 1). Positive c-MYC protein expression was associated with markers of basal-like phenotype, including cytokeratin 5/6 and epidermal growth factor receptor (EGFR; $P=0.002$ and 0.001, respectively, Table 2).

c-MYC expression was significantly expressed in breast tumours with high ATM and PI-3 kinase (both $P=0.001$, Table 2). Cell cycle regulators p16, Cyclin B1 and Cyclin E were also significantly expressed in breast tumours with positive expression of c-MYC ($P=0.018$, $P=0.023$ and $P=0.001$, respectively). Tumours with high c-MYC showed high expression of Ki67 ($P<0.001$). There was no association between c-MYC expression and BCL2, p53, p21, pAKTs473 or Retinoblastoma (Rb).

MYC expression in molecular BC subtypes. In terms of the intrinsic subtypes (PAM50), high *MYC* mRNA expression was significantly expressed in basal tumours ($P<0.001$), whereas low expression was associated with luminal A and HER2 tumours (both $P<0.001$). There was no association between *MYC* expression and luminal B tumours.

Those biomarkers that were associated with high c-MYC expression in the unselected breast tumours were further investigated in the different BC molecular classes. This showed differential association between molecular classes. In luminal A (ER+/HER2−) tumours, c-MYC expression was significantly associated with ATM ($P=0.005$), Cyclin B1 ($P=0.002$), PIK3CA ($P=0.009$) and Ki67 ($P<0.001$) but not Cyclin E nor p16 (Table 2). In contrast, in basal-like tumours, c-MYC expression was associated with positive Cyclin E ($P=0.003$) and p16 ($P=0.042$) expression but not with ATM, Cyclin B1, PI-3 kinase or Ki67 (Table 2). In HER2+ tumours, positive c-MYC expression was associated with ATM ($P=0.001$) and Ki67 ($P=0.030$) expression only.

Table 1. c-MYC expression in relation to clinicopathological parameters

	mRNA			Protein		
	Low, *n* (%)	High, *n* (%)	X^2 (*P*-value)	Negative, *n* (%)	Positive, *n* (%)	X^2 (*P*-value)
Size, cm						
<1	82 (89.1)	10 (10.9)	4.05 (0.256)	34 (50.7)	33 (49.3)	0.109 (0.991)
1–2	683 (89.2)	83 (10.8)		299 (49.6)	304 (50.4)	
>2–5	868 (86.7)	133 (13.3)		208 (49.3)	214 (50.7)	
>5	82 (83.7)	16 (16.3)		6 (46.2)	7 (53.8)	
Total	1715 (87.6)	242 (12.4)		547 (49.5)	558 (50.5)	
Grade						
1	157 (92.9)	12 (7.1)	25.85 (<**0.001**)	110 (57.3)	83 (43.0)	
2	700 (90.9)	70 (9.1)		177 (50.0)	177 (50.0)	6.72 (**0.043**)
3	794 (83.6)	156 (16.4)		258 (46.6)	296 (53.4)	
Total	1651 (87.4)	238 (12.6)		545 (49.5)	556 (50.5)	
Tubules						
1	Not available			30 (63.8)	17 (36.2)	
2				172 (47.3)	192 (52.7)	4.59 (0.101)
3				318 (49.5)	325 (50.5)	
Total				520 (49.3)	534 (50.7)	
Pleomorphism						
1	Not available			13 (54.2)	11 (45.8)	
2				225 (55.0)	184 (45.0)	8.88 (**0.012**)
3				282 (45.6)	336 (54.4)	
Total				520 (40.5)	531 (50.5)	
Mitosis						
1	Not available			203 (56.4)	157 (43.6)	
2				81 (44.3)	102 (55.7)	11.08 (**0.004**)
3				236 (46.2)	275 (53.8)	
Total				520 (49.3)	534 (50.7)	
Tumour type						
Ductal (including mixed)	Not available			437 (47.4)	484 (52.6)	
Lobular				65 (61.3)	41 (38.7)	25.48 (<**0.001**)
Medullary-like				9 (29.0)	22 (71.0)	
Other special types				31 (73.8)	11 (26.20	
Total				542 (49.3)	558 (50.7)	
Axillary nodal stage						
1	918 (88.7)	117 (11.3)		354 (52.4)	321 (47.6)	
2	275 (87.6)	39 (12.4)	2.30 (0.316)	148 (44.4)	185 (55.6)	6.14 (**0.046**)
3	536 (86.2)	86 (13.8)		43 (46.2)	50 (53.8)	
Total	1729 (87.7)	242 (12.3)		545 (49.5)	556 (50.5)	
Nottingham Prognostic Index						
⩽3.4	361 (92.3)	30 (7.7)		178 (52.5)	161 (47.5)	
>3.4	1280 (86.4)	202 (13.6)	10.12 (**0.001**)	365 (47.9)	397 (52.1)	1.99 (0.158)
Total	1641 (87.6)	232 (12.4)		543 (49.3)	558 (50.7)	
ER						
Negative	362 (77.0)	108 (23.0)	63.65 (<**0.001**)	134 (48.4)	143 (51.6)	0.25 (0.615)
Positive	1370 (90.9)	137 (9.1)		411 (50.1)	409 (49.9)	
Total	1732 (87.6)	245 (12.4)		545 (49.6)	552 (50.3)	
PgR						
Negative	791 (84.5)	145 (15.5)	15.724 (<**0.001**)	219 (49.2)	226 (50.8)	0.003 (0.959)
Positive	941 (90.4)	100 (9.6)		311 (49.1)	323 (50.9)	
Total	1732 (87.6)	245 (12.4)		530 (49.1)	549 (50.9)	
HER2						
Negative	1501 (86.7)	131 (13.3)	11.49 (**0.001**)	477 (48.4)	477 (51.6)	0.79 (0.375)
Positive	231 (94.3)	145 (5.7)		85 (52.1)	78 (47.9)	
Total	1732 (82.2)	376 (17.8)		562 (50.3)	555 (49.7)	
Triple negative						
No	1508 (90.8)	152 (9.2)	99.85 (<**0.001**)	453 (50.6)	443 (49.4)	2.93 (0.087)
Yes	224 (70.7)	93 (29.3)		84 (43.8)	108 (56.3)	
Total	1732 (87.6)	245 (12.4)		537 (49.4)	551 (50.6)	
Pam50 gene signature						
Luminal A	675 (94.4)	40 (5.6)	38.19 (<**0.001**)	Not available		
Luminal B	439 (89.8)	50 (10.2)	0.69 (0.408)			
HER2	232 (97.5)	6 (2.5)	20.92 (<**0.001**)			
Basal	227 (68.8)	103 (31.2)	162.42 (<**0.001**)			
Total	1573 (88.8)	199 (11.2)				

Abbreviations: ER = oestrogen receptor; HER2 = human epidermal growth factor receptor 2; PgR = progesterone receptor. Bold numerals signify *P*-values.

Table 2. c-MYC expression in relation to molecular breast cancer subtypes and biomarkers

	All cases			Luminal A			Basal-like			HER2+		
				c-MYC			c-MYC			c-MYC		
	Negative	Positive	X^2 (*P*-value)	Negative	Positive	X^2 (*P*-value)	Negative	Positive	X^2 (*P*-value)	Negative	Positive	X^2 (*P*-value)
ATM												
Negative	204 (55.9)	161 (44.1)	11.68 (**0.001**)	120 (58.0)	87 (42.0)	7.71 (**0.005**)	43 (46.7)	49 (53.3)	0.15 (0.697)	37 (60.7)	24 (39.3)	11.45 (**0.001**)
Positive	143 (42.9)	190 (57.1)		108 (44.8)	133 (55.2)		19 (43.2)	25 (56.8)		12 (27.3)	32 (72.7)	
Total	347 (49.7)	351 (50.2)		228 (50.9)	220 (49.1)		62 (45.6)	74 (54.4)		49 (46.7)	56 (53.3)	
PIK3CA												
Negative/ low	134 (59.8)	90 (40.2)	13.67 (**0.001**)	132 (46.8)	150 (53.2)	9.47 (**0.009**)	13 (50.0)	13 (50.0)	0.67 (0.717)	12 (63.2)	7 (36.8)	2.93 (0.302)
Medium	117 (44.7)	145 (55.3)		92 (45.5)	110 (54.5)		12 (40.0)	18 (60.0)		10 (40.0)	15 (60.0)	
High	239 (46.6)	274 (53.4)		103 (59.9)	69 (40.1)		51 (42.1)	70 (57.9)		53 (52.5)	48 (47.5)	
Total	490 (49.0)	509 (51.0)		327 (49.8)	329 (50.1)		76 (42.9)	101 (57.1)		75 (51.7)	70 (48.3)	
Cyclin B1												
Negative	200 (51.2)	191 (48.8)	5.21 (**0.023**)	136 (54.6)	113 (45.4)	9.55 (**0.002**)	28 (36.4)	49 (63.6)	0.57 (0.449)	32 (56.1)	25 (43.9)	0.56 (0.455)
Positive	138 (42.6)	186 (57.3)		83 (40.1)	124 (59.9)		24 (42.9)	32 (57.1)		27 (49.1)	28 (50.9)	
Total	338 (47.3)	377 (52.7)		219 (48.0)	237 (52.0)		52 (39.0)	81 (41.0)		59 (34.5)	112 (65.5)	
Cyclin E												
Negative	215 (52.4)	195 (47.6)	10.55 (**0.001**)	147 (49.3)	151 (50.7)	1.50 (0.220)	26 (61.9)	16 (38.1)	8.67 (**0.003**)	36 (50.0)	24 (40.0)	1.95 (0.163)
Positive	36 (34.6)	68 (65.4)		13 (38.2)	21 (61.8)		16 (31.4)	35 (68.6)		6 (40.0)	9 (60.0)	
Total	251 (48.8)	263 (51.2)		160 (48.2)	172 (51.8)		42 (45.2)	51 (54.8)		42 (56.0)	33 (44.0)	
P16												
Negative	356 (51.5)	335 (48.5)	5.62 (**0.018**)	264 (51.1)	253 (48.9)	3.37 (0.066)	37 (55.2)	30 (44.8)	4.15 (**0.042**)	47 (49.5)	48 (50.5)	0.47 (0.494)
Positive	142 (43.6)	184 (56.4)		62 (42.5)	84 (50.8)		46 (39.7)	70 (60.3)		32 (55.2)	26 (44.8)	
Total	498 (49.0)	519 (51.0)		326 (49.2)	337 (50.8)		83 (45.4)	100 (54.6)		79 (51.6)	74 (48.4)	
Ki67												
Negative	176 (59.7)	119 (40.3)	20.71 (<**0.001**)	149 (58.9)	104 (41.1)	13.45 (<**0.001**)	10 (55.6)	8 (44.4)	1.59 (0.207)	15 (71.4)	6 (28.6)	4.73 (**0.030**)
Positive	266 (43.5)	345 (56.5)		148 (43.7)	191 (56.3)		58 (40.0)	87 (60.0)		52 (49.6)	62 (54.4)	
Total	442 (48.8)	464 (51.2)		297 (50.2)	295 (49.8)		68 (41.7)	95 (58.3)		63 (48.1)	68 (51.9)	

Abbreviation: HER2 = human epidermal growth factor receptor 2. Bold numerals signify *P*-values.

MYC expression and patients' outcome. The high expression of *MYC* mRNA ($P = 0.007$, Figure 1A) and c-MYC protein ($P = 0.001$, Figure 1B) were both significantly associated with poor patient BCSS. There remained significant differences with respect to positive c-MYC expression in the survival of patients in both Stage I ($P = 0.016$) and Stage II ($P = 0.010$) (Figures 1C and D) but not Stage III ($P = 0.458$) disease (data not shown).

Moreover, there was a significant association between positive c-MYC protein expression and shorter DM-free survival in unselected breast tumours ($P = 0.017$, Figure 1E), which was maintained in luminal A tumours ($P = 0.013$, Figure 1F) but not in basal-like or HER2+ tumours (Figures 1G and H). c-MYC expression was associated with the development of bone metastases ($P = 0.019$) in unselected tumours, while in the luminal A population positive c-MYC expression was associated with liver metastases ($P = 0.039$).

In terms of adjuvant treatment, positive c-MYC expression significantly predicted a shorter DM-free survival in luminal A tumours treated with endocrine therapy ($P = 0.002$, Figure 1I) but not in patients who did not receive adjuvant therapy (Figure 1J).

Additionally, the prediction of a shorter DM-free survival in luminal A tumours treated with adjuvant endocrine therapy was observed in LN+ tumours (Figure 1K, $P = 0.012$) but not in LN− tumours (Figure 1L). In multivariate Cox regression analysis, c-MYC remained an independent predictor of a shorter DM-free survival in luminal A LN+ tumours treated with endocrine therapy ($P = 0.013$, Table 3).

Regulation of genes by MYC in biological subtypes. Top pair-wise interactions for genes probe markers associated with *MYC* expression in ER-positive and -negative tumours showed several different hubs indicated to be highly influential or regulated in the *MYC* system.

In ER-positive disease, *MYC* mRNA was significantly associated with genes implicated in translational (initiation elongation and termination) together with cellular protein metabolism (Table 4). In addition, the high expression of *MYC* was associated with the downregulation of several key hubs, including genes for translocase of outer mitochondrial membrane 40 homolog (yeast)-like (*TOMM40L*) and pre-B-cell leukaemia homeobox interacting protein 1 (*PBXIP1*; Figure 2A).

In contrast, within ER-negative disease, *MYC* mRNA was significantly associated with genes associated with glucose homeostasis, glucose metabolism and glucose transport (Table 4). *MYC* was also associated with upregulation by several genes of which calcium channel flower domain containing 1 (*CACFD1*) and proline dehydrogenase (oxidase) 1 (*PRODH*) were centralised hubs (Figure 2B).

DISCUSSION

Studies that address the prognostic significance of c-MYC protein and its influence on the response of therapeutic regimens are limited. In the current study, a large number of breast tumours were investigated for *MYC* mRNA and c-MYC protein expression in order to better understand the potential roles of this complex protein in BCs.

There is a wealth of studies that describe *MYC* amplifications in BCs, although the frequency of this amplification is highly variable (Borg *et al*, 1992; Rummukainen *et al*, 2001; Robanus-Maandag *et al*, 2003; Schlotter *et al*, 2003; Adem *et al*, 2004; Al-Kuraya *et al*, 2004; Rodriguez-Pinilla *et al*, 2007; Cancer Genome Atlas Network, 2012). In this study, the expression of *MYC* that was observed in 13.2% of breast tumours is, however, in line with the large meta-

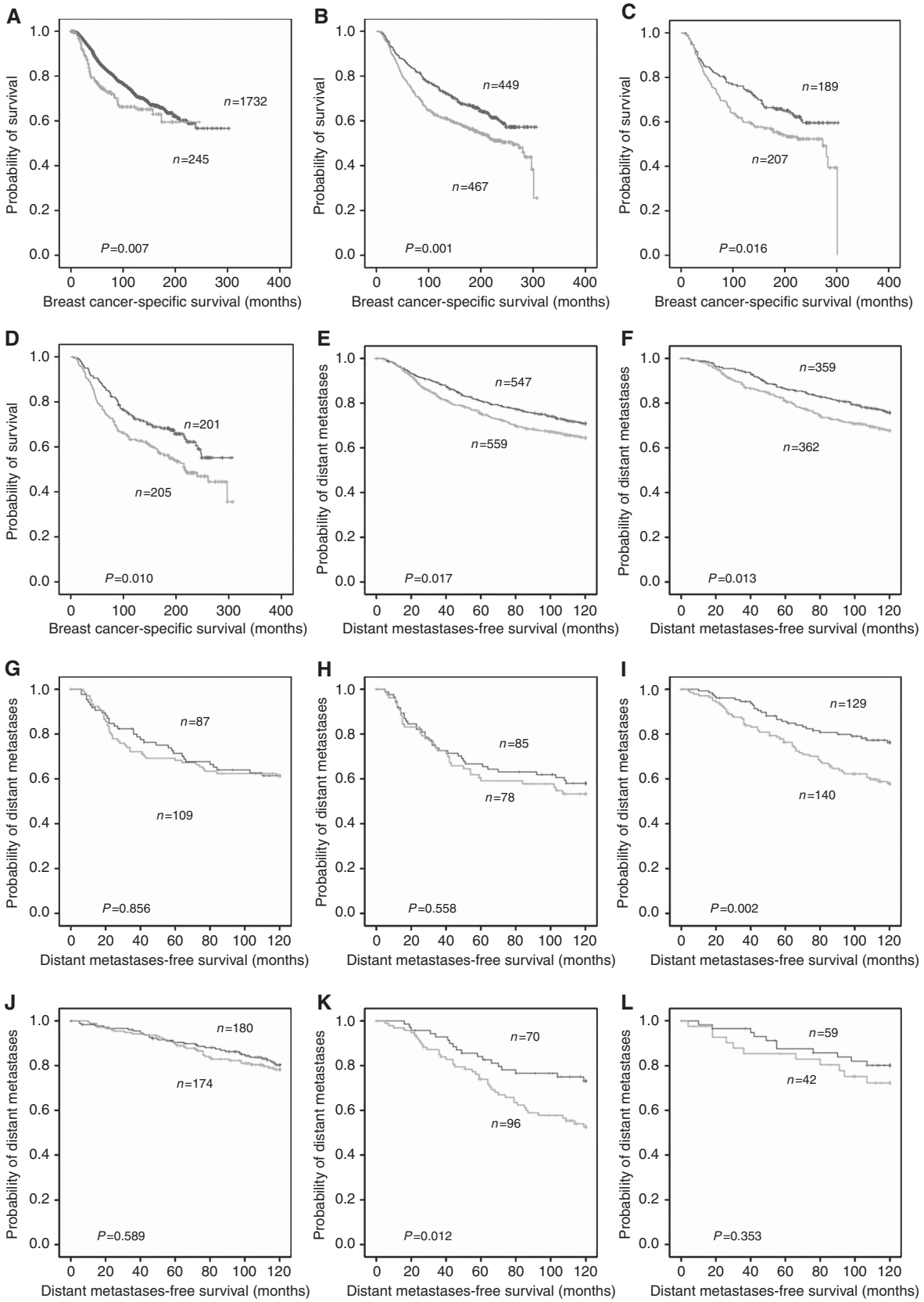

Figure 1. MYC and BC patient outcome: (**A**) *MYC* (BCSS), (**B**) c-MYC (BCSS), (**C**) c-MYC in Stage I disease (BCSS), (**D**) c-MYC in Stage II disease (BCSS), (**E**) c-MYC (DMFS), (**F**) c-MYC in luminal A tumours (DMFS), (**G**) c-MYC in basal-like tumours (DMFS), (**H**) c-MYC in HER2+ tumours (DMFS), (**I**) c-MYC in luminal A tumours treated with endocrine therapy (DMFS), (**J**) c-MYC in luminal A tumours with no adjuvant treatment (DMFS), (**K**) c-MYC in luminal A lymph node-positive patients treated with endocrine treatment, (**L**) c-MYC in luminal A lymph node-negative patients treated with endocrine treatment. Abbreviations: DMFS = distant metastases-free survival; Green = positive; blue = negative. A full color version of this figure is available at the *British Journal of Cancer* journal online.

analysis study showing amplification of *MYC* in 15.7% of BCs (Deming *et al*, 2000). Additionally, *MYC* expression was similarly associated with higher tumour grade and poor patient survival as observed with *MYC* amplification (Deming *et al*, 2000).

Table 3. Multivariate analysis of c-MYC in luminal A lymph node-positive breast tumours treated with adjuvant endocrine therapy

Parameter	Hazard ratio	95% CI	*P*-value
c-MYC	1.86	1.06–3.26	0.031
Size[a]	0.98	0.57–1.69	0.943
Grade[a]	3.64	1.96–6.76	0.015
Stage	3.64	1.96–6.76	0.001
Age (⩾50 years)	0.75	0.34–1.67	0.481

Abbreviation: CI = confidence interval.
[a]Fitted as a linear term, that is, increase in risk for change one unit in grade or size.

The expression of c-MYC protein was observed in 52.2% of invasive BCs, which is within the range of other immunohistochemical studies (45–94%) (Spandidos *et al*, 1989; Spaventi *et al*, 1994; Naidu *et al*, 2002). Although in this study the correlation between measurements of mRNA expression and those for protein expression was not perfect, MYC gene amplification has been reported to be significantly associated with overexpression of its mRNA and protein. Moreover, some authorities have reported MYC gene amplification in 15% of breast tumours, whereas mRNA level was overexpressed in 22–35% of tumours. However, up to 40% of breast tumours showed MYC protein overexpression. These figures indicate that MYC overexpression could be attributed to different mechanisms, including gene amplification, transcriptional regulation and mRNA and protein stabilisation (reviewed in Xu *et al*, 2010). Therefore, variation between gene and protein expression is likely. When tumours were stratified according to histological type, medullary-like tumours showed the highest frequency of c-MYC expression, whereas most of the lobular carcinoma showed low c-MYC expression. This is consistent with

Table 4. Top pair-wise analysis of genes associated with high *MYC* expression in ER-positive and -negative breast tumours in the METABRIC data set and pair-wise correlation cross-validation in Breast Cancer Gene-Expression Miner

Genes	Gene ontology—biological process	Correlation with *MYC* using Breast Cancer Gene-Expression Miner
ER-negative tumours		
AKT1	Glucose homeostasis; glucose metabolic process; glucose transport	$P<0.0001$ ($n=1560$)
FABP5	Glucose transport	$P<0.0001$ ($n=1353$)
FGFR4	Glucose homeostasis	$P<0.0001$ ($n=1502$)
FOXA1	Glucose homeostasis	$P<0.0001$ ($n=1526$)
IRS2	Glucose metabolic process	$P<0.0001$ ($n=1501$)
PTPN2	Glucose homeostasis	$P<0.0001$ ($n=1502$)
SEH1L	Glucose transport	$P<0.0001$ ($n=1326$)
TFAP2B	Glucose homeostasis; glucose metabolic process	$P<0.0001$ ($n=1355$)
CACFD1	Calcium ion transmembrane transport	$P<0.0001$ ($n=1282$)
PRODH	Proline catabolic process	$P<0.0001$ ($n=582$)
ER-positive tumours		
EIF4A1	Translational initiation; cellular protein metabolic process	$P<0.0001$ ($n=3834$)
EIF4H	Translational initiation	$P=0.04$ ($n=3769$)
RPL13A	Translational initiation; translational elongation; cellular protein metabolic process; translational termination	$P<0.0001$ ($n=3427$)
RPL17	Translational initiation; translational elongation; cellular protein metabolic process; translational termination	$P<0.0001$ ($n=3654$)
RPL18A	Translational initiation; translational elongation; cellular protein metabolic process; translational termination	$P<0.0001$ ($n=3449$)
RPL8	Translational initiation; translational elongation; cellular protein metabolic process	$P<0.0001$ ($n=3710$)
RPL9	Translational initiation; translational elongation; cellular protein metabolic process; translational termination	$P<0.0001$ ($n=3453$)
RPLP0	Translational initiation; translational elongation; cellular protein metabolic process; translational termination	$P<0.0001$ ($n=3518$)
RPS5	Translational initiation; translational elongation; cellular protein metabolic process; translational termination	$P<0.0001$ ($n=3653$)
EEF1B2	Translational elongation; cellular protein metabolic process	$P<0.0001$ ($n=3810$)
ATF3	Cellular protein metabolic process	$P<0.0001$ ($n=3714$)
CCT7	Cellular protein metabolic process	$P<0.0001$ ($n=4056$)
HSPD1	Cellular protein metabolic process	$P<0.0001$ ($n=3454$)
TUBB6	Cellular protein metabolic process	$P<0.0001$ ($n=3835$)
TOMM40L	Ion, protein and transmembrane transport	$P<0.0001$ ($n=2418$)
PBXIP1	Cell differentiation; negative regulation of transcription	$P<0.0001$ ($n=3875$)

Abbreviations: ER = oestrogen receptor; METABRIC = Molecular Taxonomy of Breast Cancer International Consortium.

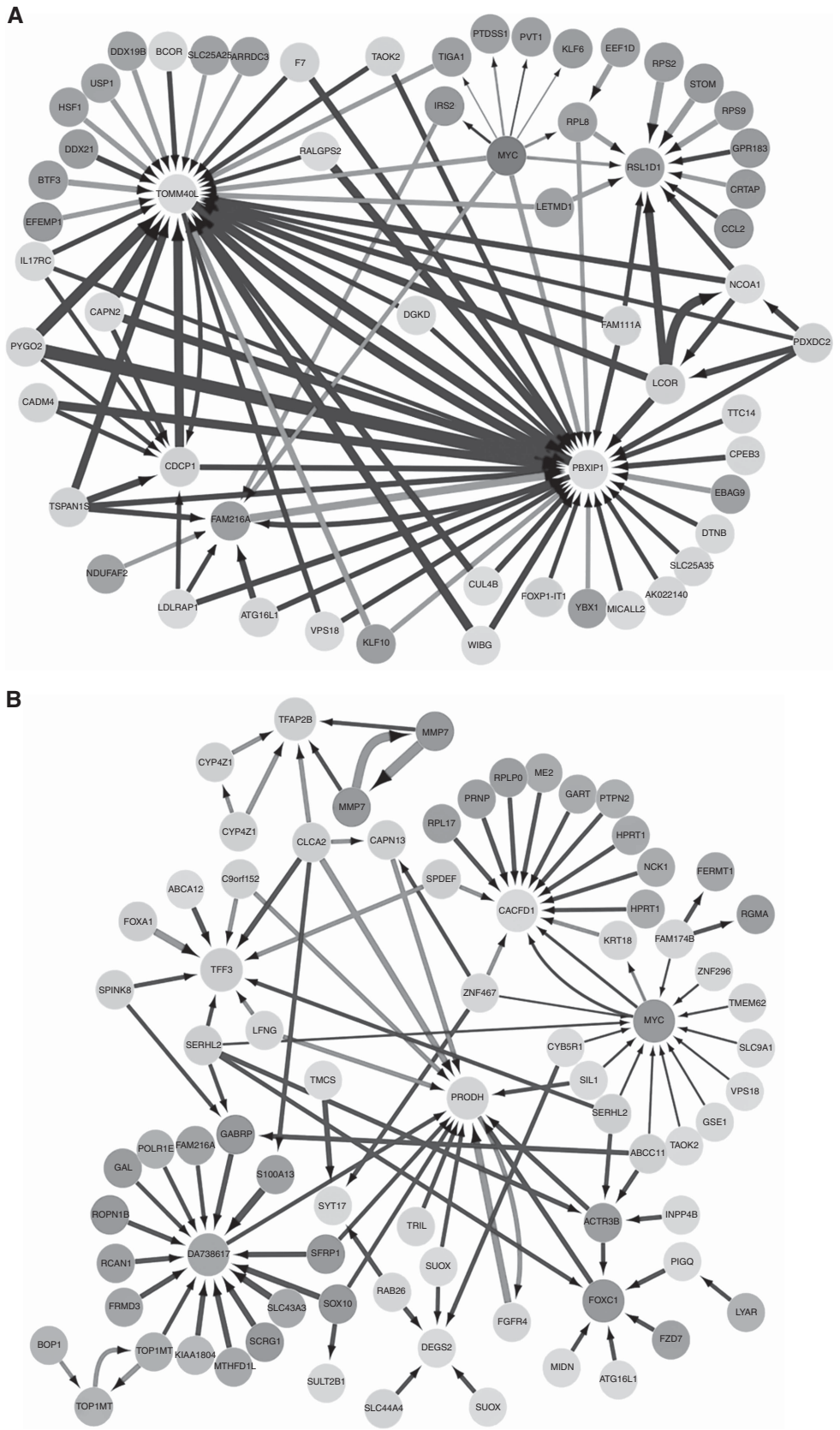

Figure 2. Gene interactions with MYC. Top pair-wise interactions for genes associated with *MYC* expression show hubs that are indicated to be highly influential or regulated in the *MYC* system in (**A**) ER-positive and (**B**) ER-negative disease. Each gene probe is represented by a node and the interaction weight between them as an edge, the width being defined by the magnitude of the weight. Interactions are directed from a source gene to a target gene as indicated by arrows. Red interactions indicate an excitatory interaction and blue indicates an inhibitory interaction. Red node gradient represents a positive fold change and green node gradient represents a negative fold change in the high *MYC* class. A full color version of this figure is available at the *British Journal of Cancer* journal online.

previous studies that observed rare *MYC* amplification/c-MYC protein overexpression in lobular carcinomas, whereas it is more frequent in non-lobular breast carcinomas (Spaventi *et al*, 1994; Janocko *et al*, 2001).

In this study, high expression of MYC was associated with higher tumour grade and the basal-like phenotype. This is in agreement with previous clinicopathological studies (Deming *et al*, 2000; Liao and Dickson, 2000; Rummukainen *et al*, 2001; Naidu *et al*, 2002; Schmitt and Reis-Filho, 2002; Shanmugham *et al*, 2004; Rodriguez-Pinilla *et al*, 2007; Cancer Genome Atlas Network, 2012). However, there was a lack of significant relation with tumour size or hormone receptor status; absence of these relationships have been demonstrated in previous studies (Spandidos *et al*, 1989; Naidu *et al*, 2002). These findings are not surprising in view of the multifunctional nature of MYC oncogene, with pivotal roles in proliferation, differentiation and cell death.

In the whole cohort, high *MYC* and c-MYC expression was associated with poor patient survival. Additionally c-MYC predicted a shorter metastatic-free survival, and this significance was maintained in multivariate analysis independently of tumour grade, nodal stage and size. Moreover, the relation between c-MYC and the development of resistance to antioestrogen treatment has been addressed in many studies (Santos *et al*, 1988; Venditti *et al*, 2002; McNeil *et al*, 2006; Planas-Silva *et al*, 2007; Musgrove *et al*, 2008) and is in agreement with our observations in relation between high c-MYC expression and shorter DM-free survival in those patients who received hormonal treatment and was an independent prognostic indicator for such group. Unlike Roux-Dosseto *et al* (1992), there was no significant association between tumour relapse and c-MYC expression in lymph node-negative patients. c-MYC protein may affect the response to chemotherapy probably through DNA damage response regulation (Berns *et al*, 1992a,b; Nass and Dickson, 1997; Gewirtz *et al*, 1998; Aulmann *et al*, 2006) although in the current study we found no evidence for this. Interestingly, *MYC* amplification in colon carcinoma predicts better response to 5-FU adjuvant chemotherapy (disease-free and cancer-specific survivals have been improved by 30%) (Arango *et al*, 2001; Barratt *et al*, 2002) but only in p53 wild-type tumours. Although this type of study in BCs has not been published yet, Rakha *et al* (2007) described improvement of the poor prognosis of basal subtype of triple-negative BCs by treatment with chemotherapy. These tumours often have amplified EGFR gene (Reis-Filho *et al*, 2005) and according to the described *MYC* co-amplification in BCs (Al-Kuraya *et al*, 2004; Miura *et al*, 2008), and the association between c-MYC expression and basal-like BCs, we may hypothesise the possibility that c-MYC overexpression may contribute to favourable response to chemotherapy, specially whose which contain 5-FU. Suppression of C-MYC transcription in BC cells after 5-FU treatment supports the direct effect of 5-FU on the oncogene activity, probably mediated by upstream signalling inhibition (Hernandez-Vargas *et al*, 2006).

We observed that c-MYC expression in luminal A tumours was associated with negative/low PI-3 kinase expression. Indeed, using a mouse BC model, PIK3CA-driven recurrent tumours with *MYC* amplification can lead to the inactivation of PIK3CA and remain independent of the PI-3 kinase pathway resulting in tumours developing resistance to PI-3 kinase pathway-specific targeted therapies (Liu *et al*, 2011). This could suggest that luminal A tumours, compared with other biological subtypes of BCs, are more susceptible to PI-3 kinase inhibitor resistance. Within luminal A tumours, high c-MYC expression was also associated with an increase in cell cycle activity indicated by high expression of Cyclin B1 and Ki67. Regulation of cyclin B1 is essential for the initiation of mitosis as it regulates the G2–M transition of the cell cycle. MYC regulates the CCNB1 gene promoter and the high expression of Cyclin B1 seen in the luminal A tumours, which has previously been associated with poor prognosis of hormone receptor-positive BCs (Hu *et al*, 2006; Agarwal *et al*, 2009), is most likely as a consequence of this regulation.

In basal-like tumours, c-MYC expression was associated with Cyclin E and the cyclin-dependent kinase inhibitor p16. p16 has previously been associated with ER-negative BCs and poor patient outcome particularly those treated with adjuvant chemotherapy (Dublin *et al*, 1998; Han *et al*, 2001; Nemtsova *et al*, 2007). Both cyclin-E and p16 are inextricably linked with the G1–S-phase transition of the cell cycle. c-MYC is an upstream activator of cyclin-E/CDK2 activity and promotes cell cycle progression from G1 to S phase independent of the Rb pathway and can compensate for low cyclin D/cdk4 kinase activity (Gray-Bablin *et al*, 1996; Alevizopoulos *et al*, 1997), whereas p16 arrests this transcriptional activity at the G1 phase. Rb is thought to promote an aggressive form of triple-negative BCs with *MYC* overexpression, although in the current study we found no evidence that RB was associated with c-MYC expression in any BC biological subtype (Knudsen *et al*, 2015). The p16 protein has a very long half-life (Sherr, 1996) and therefore accumulates in the cells with increasing number of cell cycles. This might partly explain high p16 expression in tumours with high growth fraction.

Although co-amplification of *MYC* and *HER2* has previously been associated with very poor patient outcome in BCs by promoting a stem-like phenotype (Nair *et al*, 2014), we found no evidence to suggest this particularly as there was a negative relationship between *MYC* expression and HER2 status. We did, however, observe that in HER2+ tumours there was a significant relationship between c-MYC protein and high cell proliferation, as determined by Ki67, suggesting that co-expression is linked with more aggressive tumours. Conversely, there was also high correlation with the co-expression of c-MYC and ATM in HER2+, suggesting that, while these tumours are highly proliferative, they are also undergoing higher levels of cell cycle arrest, DNA repair or apoptosis by MYC activating ATM-dependent checkpoint responses (Guerra *et al*, 2010).

The mechanisms of *MYC* were further explored in ER-positive and -negative tumours using ANN analysis of the METABRIC data. Here we show that *MYC* is potentially driving glucose metabolism in ER-negative, but not in ER-positive, tumours providing necessary energy required for cell proliferation (the Warburg effect), a key feature of these poor prognostic tumours. It is well-documented that c-MYC regulates glucose metabolism and glutamine uptake and contributes to the metabolic preprogramming required for cancer cells to adapt to the tumour microenvironment (for reviews, see Li and Simon, 2013; Wahlstrom and Arsenian Henriksson, 2015). Our data confirm previous studies in triple-negative breast tumours where those with *MYC* gene copy gain have increased glucose uptake (Palaskas *et al*, 2011) and the expression of thioredoxin-interacting protein is downregulated driving the activation of glucose metabolism (Shen *et al*, 2015). A mechanistic association between MYC and glucose metabolism in endocrine resistance in BCs has also recently been described (Shajahan-Haq *et al*, 2014).

In ER-positive tumours, *MYC* was associated with translational function, particularly the family of ribosomal subunit proteins and eukaryotic translation-initiation factors. Increases in rRNA synthesis, ribosomal biogenesis and translational initiation/elongation are common cancer features, which can be driven by MYC (for a review, see Ruggero, 2009), and we show that enhanced ribosome assembly and transcriptional activity is a probable feature of luminal BCs.

CONCLUSIONS

Although MYC is multifunctional, it is apparent that different biological pathways are predominant in the separate molecular

subtypes of BCs. The diverse mechanisms of c-MYC function as potential therapeutic targets, particularly in BC subtypes, therefore warrants further investigation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

ARG conceived the study, participated in its design and analysis and drafted the manuscript. MA participated in the analysis and interpretation; SE, CCN and MDR conducted the immunohistochemical studies; CC carried out the molecular genetic studies; DA and GB carried out statistical and ANN-based bioinformatics analysis; SM, RDM, IOE and EAR participated in its design, analysis and interpretation. All authors read and approved the final manuscript.

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