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ORIGINAL ARTICLE

In vitro and *in vivo* analysis of *B-Myb* in basal-like breast cancer

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A defining feature of basal-like breast cancer, a breast cancer subtype with poor clinical prognosis, is the high expression of 'proliferation signature' genes. We identified *B-Myb*, a MYB family transcription factor that is often amplified and overexpressed in many tumor types, as being highly expressed in the proliferation signature. However, the roles of B-Myb in disease progression, and its mammary-specific transcriptional targets, are poorly understood. Here, we showed that *B-Myb* expression is a significant predictor of survival and pathological complete response to neoadjuvant chemotherapy in breast cancer patients. We also identified a significant association between the G/G genotype of a nonsynonymous *B-Myb* germline variant (rs2070235, S427G) and an increased risk of basal-like breast cancer [OR 2.0, 95% CI (1.1–3.8)]. In immortalized, human mammary epithelial cell lines, but not in basal-like tumor lines, cells ectopically expressing wild-type *B-Myb* or the S427G variant showed increased sensitivity to two DNA topoisomerase IIα inhibitors, but not to other chemotherapeutics. In addition, microarray analyses identified many G2/M genes as being induced in *B-Myb* overexpressing cells. These results confirm that *B-Myb* is involved in cell cycle control, and that its dysregulation may contribute to increased sensitivity to a specific class of chemotherapeutic agents. These data provide insight into the influence of *B-Myb* in human breast cancer, which is of potential clinical importance for determining disease risk and for guiding treatment.
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Keywords: *B-Myb*; MYBL2; breast cancer; basal-like

Introduction

Breast cancer is not a single disease, but rather, represents at least six subtypes (Perou *et al.*, 2000; Sorlie *et al.*, 2003; Hu *et al.*, 2006). These include luminal A, luminal B, normal-like, human epidermal growth factor receptor 2 (HER2)-enriched, claudin-low and basal-like, each with unique gene expression profiles and distinct clinical outcomes. The majority of breast cancer cases (60–80%) comprise the luminal/estrogen receptor-α positive (ERα+) tumors, whereas basal-like breast carcinoma accounts for 10–15% of all cases. The basal-like subtype, often clinically observed as 'triple-negative' tumors (negative for ERα, PR and HER2), is of particular interest because treatment options are limited to chemotherapy only and patients with this disease typically have poor outcomes. One contribution to the poor outcome of basal-like breast cancer patients may be their high cellular proliferation rates, which is manifested transcriptionally as the high expression of the 'proliferation signature'; this is a dominant gene signature that is a marker of cell proliferation rates across multiple tumor types (Hu *et al.*, 2006; Whitfield *et al.*, 2006). *B-Myb*, a gene with known cell cycle control functions and implications in tumorigenesis (Sala, 2005), is one of approximately 100 genes that define the proliferation signature.

B-Myb is a member of the vertebrate MYB family of nuclear transcription factors. In humans, this family is comprised of *A-Myb* (MYBL1), *B-Myb* (MYBL2) and *c-Myb* (MYB). Each family member is able to recognize and bind to the same DNA consensus sequence (PyAAC(G/T)G) to promote gene transcription; however, varying tissue-specific expression patterns, as well as protein–protein interactions with unique co-factors, suggest that distinct biological roles exist for each MYB family member (Rosinski and Atchley, 1998; Sala, 2005). MYB proteins are encoded in the genomes of both plants and animals and control processes from flavonoid production to cellular proliferation (Rosinski and Atchley, 1998; Ito *et al.*, 2001). In contrast to vertebrates, invertebrates contain only one MYB protein, which in Drosophila (*dMYB*) is phylogenetically and functionally complementary to vertebrate B-Myb, suggesting B-Myb to be the most ancient family member (Davidson *et al.*, 2005). The expression of *B-Myb*, unlike *c-Myb* and *A-Myb* (Mucenski *et al.*, 1991; Trauth *et al.*, 1994; Toscani *et al.*, 1997; Ness, 2003; Malaterre *et al.*, 2007), is ubiquitously expressed in virtually all proliferating cells as a regulator of cell cycle progression and plays an essential role in

vertebrate development. Knocking out murine *B-Myb* causes early embryonic lethality (E4.5–6.5) resulting from unsuccessful inner cell mass formation (Tanaka *et al.*, 1999).

MYB family members have been implicated in tumorigenesis for several decades. The *c-Myb* proto-oncogene was first identified as the mammalian homolog of *v-Myb*, which is the transforming gene transmitted by the avian myeloblastosis and E26 retroviruses causing acute leukemia in birds (Klempnauer *et al.*, 1982; Leprince *et al.*, 1983). *A-* and *B-Myb* were later discovered during low-stringency screening of human complementary DNA libraries (Nomura *et al.*, 1988). The *B-Myb* chromosomal locus, 20q13, is amplified and/or highly expressed in a variety of tumor types, including breast, prostate, liver and ovarian carcinomas, and in most cases this high expression portends a poor prognosis (Sala, 2005). *B-Myb* is also an important marker of poor outcome in embryonal tumors of the central nervous system (Pomeroy *et al.*, 2002). Recently, a nonsynonymous *B-Myb* germline variant (rs2070235) causing a serine to glycine amino acid change (S427G) was linked to a decrease in overall cancer risk for neuroblastomas, chronic myelogenous leukemia and colon cancers in a combined dataset of cases and controls (Schwab *et al.*, 2007). However, the molecular roles of *B-Myb* in disease progression, as well as its transcriptional target genes in the mammary gland, are still poorly understood. To gain insight into *B-Myb* and its involvement in breast cancer, we analysed the expression of *B-Myb* across the breast cancer subtypes, examined its relationship to survival and pathological complete response, and the correlation of variant rs2070235 to disease risk. We also manipulated the expression of *B-Myb* and the S427G variant in normal and tumor-derived mammary cell lines and observed alterations in drug sensitivity and cell cycle profiles.

Results

High B-Myb *expression in breast tumors predicts poor outcome*

To assess the relevance of *B-Myb* gene expression across the breast cancer subtypes, breast tumor microarray data from the Netherlands Cancer Institute (NKI-295, $n = 295$, van de Vijver *et al.*, 2002) were analysed. Tumor samples were classified into five breast cancer subtypes using a single-sample centroid-based predictor as described (Hu *et al.*, 2006). An ANOVA (analysis of variance) analysis performed on these stratified samples showed that *B-Myb* expression differs significantly across the subtypes and was highest in basal-like tumors (Figure 1).

To test for correlations between *B-Myb* mRNA expression alone and patient outcome, we analysed the NKI patients who were not receiving adjuvant systemic treatment (that is, local treatment only; $n = 165$). This allowed us to identify the prognostic abilities of *B-Myb* better, without the confounding data of treatment
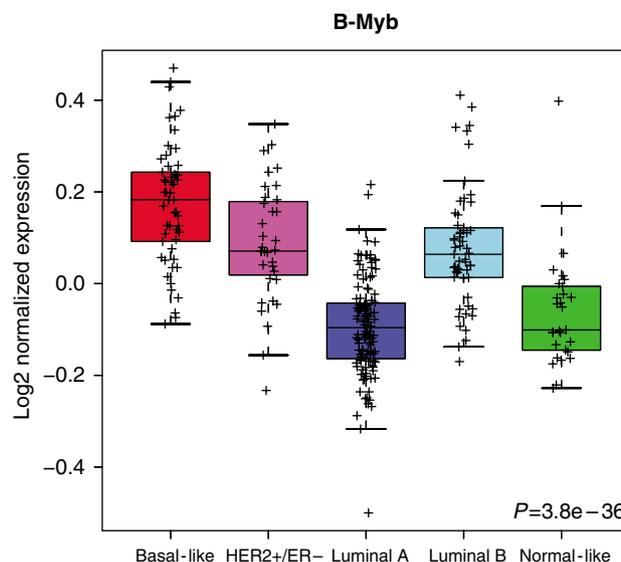

Figure 1 *B-Myb* expression across breast cancer subtypes. The NKI breast tumor microarray dataset ($n = 295$) was classified into the five intrinsic subtypes and box plots used to visualize *B-Myb* expression according to breast cancer subtypes. Statistical significance was calculated by ANOVA. HER2, human epidermal growth factor receptor 2; ER, estrogen receptor.

response. The NKI 'local-only' tumors were rank ordered into halves (low/high) on the basis of their *B-Myb* expression levels and analysed for overall survival (OS) and relapse-free survival (RFS) by Kaplan–Meier analysis. Poor overall survival and RFS were highly correlated ($P < 0.001$) with *B-Myb* high expression levels in these NKI samples (Figure 2a, and RFS data not shown). *B-Myb* expression alone was also able to significantly predict overall survival on local-only-treated luminal A subtype tumors ($n = 72$) (Figure 2b), luminal B subtype tumors ($n = 26$) (Figure 2c) and HER2 +/ER− subtype tumors ($n = 21$) (Figure 2d), but not basal-like tumors ($n = 30$) (Supplementary Figure 1A). We then evaluated the prognostic ability of *B-Myb* using two other published breast tumor microarray datasets (Miller *et al.*, 2005; Wang *et al.*, 2005). Wang *et al.*'s (2005) study ($n = 286$) consisted of microarrays on untreated, lymph-node-negative primary ER + and ER− breast cancers with relapse data, and *B-Myb* was capable of predicting RFS in these patients (Figure 2e). On this same dataset, *B-Myb* also predicted RFS in the ER + patient subset ($n = 209$), but not in the ER− subset ($n = 77$) (Supplementary Figures 1B and C). Another dataset consisting of primary invasive tumors (Miller *et al.*, 2005) ($n = 234$) was tested and similar results were found (Figure 2f).

To determine whether *B-Myb* expression was involved with pathological complete response, we used the data of Hess *et al.* (2006), where microarrays were performed on pre-treatment breast tumors from patients receiving neoadjuvant paclitaxel, followed by 5FU-adriamycin-cyclophosphamide (T/FAC; $n = 133$). Again, samples were split into two groups on the basis of *B-Myb* expression (low/high). *B-Myb* high expression was again
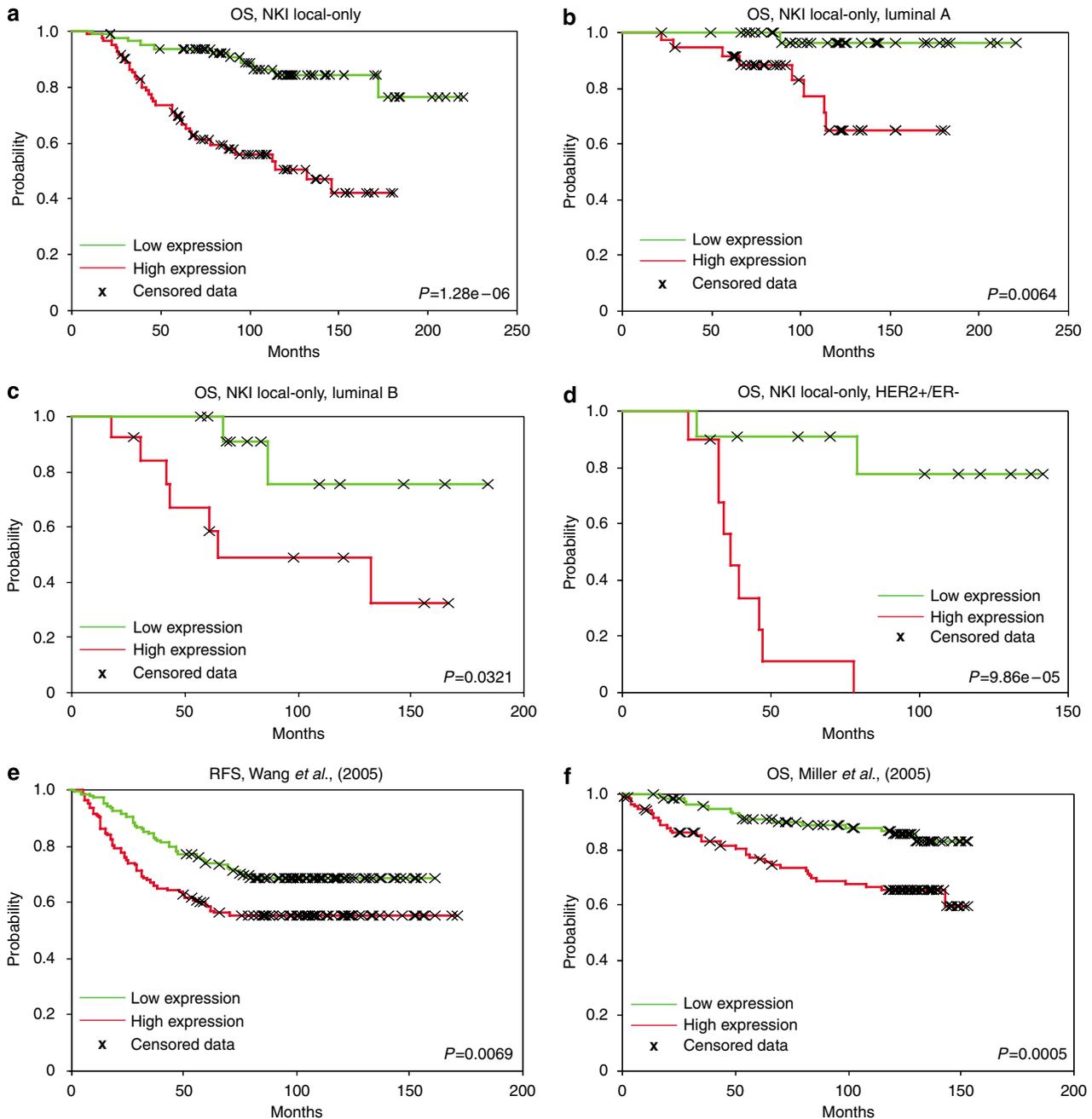
Figure 2 High expression of *B-Myb* correlates with poor outcome. Kaplan–Meier survival analyses based on *B-Myb* expression values rank ordered into halves (low/high). (**a**–**d**) Overall survival (OS) of locally treated NKI tumor samples: (**a**) All subtypes combined ($n=165$), (**b**) luminal A ($n=72$), (**c**) luminal B ($n=26$), (**d**) HER2 + /ER− ($n=21$). (**e**) RFS, Wang *et al*. (2005) ($n=286$), a locally treated, lymph-node-negative tumor microarray dataset. (**f**) Miller *et al*. (2005) breast tumor microarray dataset ($n=234$). RFS, relapse-free survival; HER2, human epidermal growth factor receptor 2; ER, estrogen receptor.

associated with subtype (data not shown) and also with pathological complete response, as calculated by χ^2 test ($P=0.008$; Supplementary Table 1).

B-Myb *germline variant (rs2070235) increases the risk of basal-like breast cancer*
A nonsynonymous germline *B-Myb* variant exists that causes a serine to glycine substitution (rs2070235,

S427G). This non-conservative change prompted us to look for correlations between this variant and baseline susceptibility risk in the population-based Carolina Breast Cancer Study (CBCS) (Newman *et al*., 1995). Odds ratios for the *B-Myb* genotype and all breast cancer cases, luminal A, and basal-like cases, versus controls are presented in Table 1. There was no association between *B-Myb* genotype and all breast cancer cases ($P=0.71$); however, a statistically significant

Table 1 *B-Myb* variant genotype (G/G) increases the risk of basal-like breast cancer

B-Myb genotype	Controls (N = 1814)	Basal-like cases (N = 206)	OR (95% CI)[a]	Luminal A cases (N = 698)	OR (95% CI)[a]	All cases (N = 1256)	OR (95% CI)[a]
A/A	1319	131	Referent	531	Referent	912	Referent
A/G	436	60	1.2 (0.8–1.7)	149	0.8 (0.7–1.1)	301	1.0 (0.8–1.2)
G/G	59	15	2.0 (1.1–3.8)	18	0.8 (0.5–1.4)	43	1.0 (0.6–1.5)
Trend test			P = 0.047		P = 0.14		P = 0.71

Abbreviations: A, wild type; G, variant; OR, odds ratio. Odds ratios for the *B-Myb* genotype, and basal-like, luminal A and all breast cancer cases. Patient samples were genotyped for the *B-Myb* polymorphism at codon 427, rs2070235.
[a]Adjusted for offsets, age and race.

association was observed for basal-like breast cancer ($P = 0.047$), but not for luminal A ($P = 0.14$). No association was observed for *B-Myb* genotype and other breast cancer subtypes (luminal B, HER2+/ER− and unclassified: data not shown). Odds ratios were similar in African-Americans and Caucasians (likelihood-ratio tests for interaction with race were not statistically significant). Among controls in the CBCS, allele frequencies for the *B-Myb* G allele were higher in African-Americans (0.27) than in Caucasians (0.08).

Ectopic expression of B-Myb *increases sensitivity to topoisomerase IIα inhibitors*
Given *B-Myb*'s expression within the proliferation signature, evidence suggesting that rapidly growing tumors may be more chemotherapy-sensitive and also the correlation between *B-Myb* expression and pathological complete response, we sought to determine whether ectopic expression of *B-Myb in vitro* had an effect on sensitivity to chemotherapeutics. *B-Myb* and the *B-Myb* S427G variant were overexpressed in two *hTERT*-immortalized human mammary epithelial cell lines (HME-CC, ME16C) and two basal-like tumor-derived lines (SUM102, SUM149) (Supplementary Figure 2). Low endogenous B-Myb levels were detectable by western blot in the tumor lines, and in all cell lines by microarray analysis for mRNA levels (Supplementary Figure 2 and data not shown). All cell lines were also genotyped for rs2070235 and identified as homozygous for the major allele. It is important to note that the two normal tissue-derived cell lines have a basal-like phenotype when assessed by gene expression analysis (Troester *et al.*, 2004); thus, these lines represent appropriate counterparts to the two basal-like tumor lines.

Cells ectopically expressing *B-Myb*, or S427G variant, were treated with a panel of chemotherapy agents, including two DNA topoisomerase IIα (TOP2A) inhibitors (doxorubicin, etoposide), a DNA topoisomerase I inhibitor (camptothecin), a microtubule stabilizer (paclitaxel), a DNA alkylating agent (carboplatin) and an antimetabolite (5-fluorouracil), most of which are commonly used in breast cancer treatment. HME-CC cells overexpressing *B-Myb*, or the S427G variant, were approximately twice as sensitive to the two DNA TOP2A inhibitors, on the basis of IC50 assays, compared with the parental cell lines, but showed no significant change in sensitivity to the other drugs

(Figure 3a). To further test *B-Myb* effects on chemotherapy sensitivity, another immortalized HMEC line (ME16C) and two basal-like tumor-derived cell lines (SUM102, SUM149) were tested. The ME16C cell line was also sensitized to TOP2A inhibitors by *B-Myb* expression, but not to treatment with other chemotherapeutics (Figure 3b and data not shown); however, this sensitivity profile was not observed in either of the two basal-like tumor-derived lines (Figures 3c and d), where *B-Myb* expression had no apparent effect. The *B-Myb* S427G variant was also tested in each cell line for chemosensitivity, and it behaved similarly as the cells overexpressing wild-type *B-Myb* (Figure 3a and data not shown).

Gene expression analysis of cell lines ectopically expressing B-Myb
To look for gene expression changes in cells overexpressing *B-Myb*, and to further assess the chemotherapy phenotype, microarrays were performed on the HME-CC cell lines. Under normal, non-confluent conditions, the only statistically significant expression difference (significance analysis of microarrays (SAM) analysis (Tusher *et al.*, 2001) with a 3% false discovery rate) was in *B-Myb* itself; also, no gene expression differences were observed between the *B-Myb* and *B-Myb* S427G-expressing cell lines. As a chemotherapy-related phenotype was observed in TOP2A inhibitors, we tested the cell lines after treatment with the 72-h IC50 dose of doxorubicin. In a two-class SAM analysis (that is, doxorubicin-treated HME-CC control versus doxorubicin-treated HME-CC + *B-Myb*), 217 genes were identified (false discovery rate <3%; Supplementary Table 2).

An EASE (Expression Analysis Systematic Explorer) analysis (Hosack *et al.*, 2003) was performed and many cell cycle-related gene ontology categories were identified as being significantly enriched (Table 2). Therefore, in doxorubicin-treated cells overexpressing *B-Myb*, there was a significantly higher expression of many cell cycle genes compared with the treated control.

B-Myb is thought to be a transcriptional regulator of G2/M genes (Zhu *et al.*, 2004). To determine whether the 217-gene SAM list was enriched for genes within a particular phase of the cell cycle, each gene was assigned to a specific phase by comparing it with a known list derived from a precise cell cycle microarray time-course experiment (Whitfield *et al.*, 2002), or by literature search. Out of 217 significant genes, 101 genes were
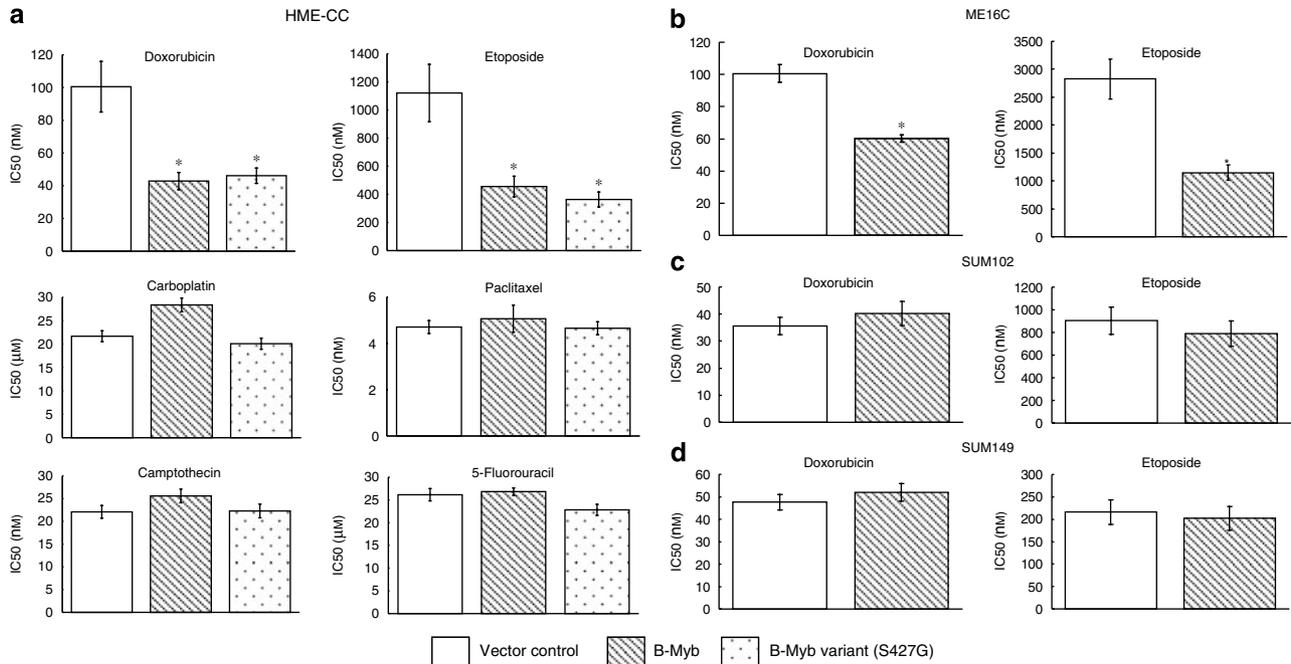
Figure 3 Drug sensitivities in *B-Myb*-overexpressing cell lines. IC50 doses (72 h) of chemotherapy on cell lines stably expressing vector control, *B-Myb* or *B-Myb* S427G variant. Each MTT experiment was performed in triplicate and error bars represent 95% confidence intervals (*$P < 0.001$ relative to vector control). (**a**) *hTERT*-immortalized HMEC line HME-CC. (**b**) *hTERT*-immortalized HMEC line ME16C. (**c**) Basal-like tumor-derived cell line SUM102 and (**d**) SUM149. HMEC, human mammary cell; *hTERT*, human telomerase reverse transcriptase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide.

Table 2 Top 15 significant gene ontology categories determined by EASE analysis for *B-Myb*-associated genes

			Doxorubicin-induced genes		
Gene ontology category	*List hits*	*List total*	*Population hits*	*Population total*	*Bonferroni P-value*
Mitotic cell cycle	58	138	352	13248	2.59E−51
Cell cycle	65	138	745	13248	8.60E−41
M phase	37	138	174	13248	1.22E−34
Nuclear division	35	138	167	13248	2.84E−32
Mitosis	32	138	131	13248	2.31E−31
M phase of mitotic cell cycle	32	138	133	13248	3.87E−31
Cell proliferation	65	138	1116	13248	4.33E−30
DNA replication and chromosome cycle	29	138	199	13248	2.21E−21
DNA metabolism	36	138	579	13248	4.60E−15
DNA replication	20	138	156	13248	1.22E−12
S phase of mitotic cell cycle	20	138	158	13248	1.54E−12
Spindle	17	135	97	12954	1.66E−12
Regulation of cell cycle	28	138	406	13248	4.68E−12
Cytokinesis	16	138	112	13248	3.14E−10
Cell growth and/or maintenance	81	138	3996	13248	3.44E−09

Abbreviations: EASE, Expression Analysis Systematic Explorer. Significant genes, as determined by SAM, for doxorubicin-treated *B-Myb*-overexpressing HME-CC cells versus doxorubicin-treated controls were input to EASE and analysed for enriched gene ontology categories.

identified as being specifically induced during the cell cycle and 60 out of 101 genes were assigned as G2/M-specific genes (Figure 4). In addition, earlier known B-Myb target genes were present on the SAM list, including *CDC2*, *Cyclin B1*, *BIRC5* and the B-Myb binding partner, *LIN-9*.

Cell cycle profiles of B-Myb-*overexpressing cells*
As *B-Myb*-expressing, doxorubicin-treated cells produced a significant G2/M-enriched gene list, we

hypothesized that the cell cycle profiles of these cells may be different from those of treated controls. *B-Myb*-expressing HME-CC and empty vector controls were both treated with a range of doxorubicin doses for 48 h and their cell cycle profiles analysed for DNA content using flow cytometry. At zero dose or a high dose of doxorubicin, the cell cycle profiles for both *B-Myb*-overexpressing cells and controls were identical in terms of the percentage of cells in G1 and G2/M phase (Figure 5). However, at low and intermediate concentrations (10–35 nM) of doxorubicin there was a significant

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difference in the number of cells in G1 or G2/M phase, with a larger percentage of *B-Myb*-overexpressing cells in G1 phase versus controls, and a lower percentage of *B-Myb*-overexpressing cells in G2/M phase (Figures 5a and b, respectively); very similar results were obtained with etoposide treatment (Supplementary Figure 3). At high doses of doxorubicin or etoposide, regardless of *B-Myb* expression, the majority of cells were arrested in G2/M phase.

Discussion

Clinically defined as ER−, PR− and HER2 not amplified, the basal-like subtype of breast cancer portends a poor prognosis. Basal-like breast cancer has an inherently high proliferation rate, which is identified by high
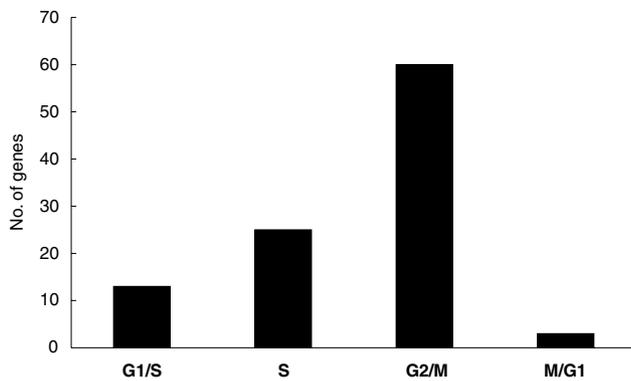

Figure 4 Enrichment of G2/M phase genes in doxorubicin-treated *B-Myb*-overexpressing HME-CC cells. Significance analysis of microarrays was used to identify the 217 significant genes that had high expression in *B-Myb*-overexpressing cells. These genes were then assigned to a specific phase of the cell cycle by comparing them with Whitfield *et al.*'s study (2002), which identified 101 out of 217 genes as being specifically induced during the cell cycle. The graph shows to which phase of the cell cycle these 101 genes mapped.

expression of the 'proliferation signature' genes through microarray analysis; this signature has been identified in many tumor types, as per several publications, and is highly enriched for cell cycle-regulated genes (Whitfield *et al.*, 2006). Here, we find that *B-Myb*, a gene highly expressed within the proliferation cluster, plays an important role in regulating cell cycle progression, which likely has effects on patient prognosis and response to chemotherapy.

We showed that *B-Myb* high expression was significantly associated with the poor outcome, basal-like breast cancer subtype, and that *B-Myb* gene expression levels alone predicted poor outcomes in the absence of therapy (that is, prognosis, Figure 2), and are correlated with achieving a pathological complete response (that is, prediction, Supplementary Table 1). Stratification of *B-Myb* expression, even within the luminal A, B and HER2+/ER− subtypes, or ER+ tumors, was significantly associated with survival. Stratification of *B-Myb* expression within basal-like tumors did not predict outcomes, nor was outcome predicted in the ER− subset of the Wang *et al.* (2005) dataset (which includes basal-like tumors); however, these are inherently poor-outcome tumors that significantly tend towards high *B-Myb* expression (Figure 1).

The *B-Myb* chromosomal locus, 20q13, is found amplified in a variety of cancers, including breast (Chin *et al.*, 2006), and this amplification is linked to poor prognosis (Bergamaschi *et al.*, 2006). Gene copy number analysis across breast tumor subtypes suggested that the genomic amplification of *B-Myb* was not enriched in the basal-like subtype, but instead was enriched in the luminal B subtype (Bergamaschi *et al.*, 2006). Therefore, within luminal tumors, *B-Myb* amplification and expression is an event that seems to be selected for and portends a poor prognosis (Figures 2b and c). In basal-like tumors, the high expression of *B-Myb* may be because of other regulatory mechanisms, possibly by virtue of their inherently high proliferation rates, by amplification of transcription factor(s) targeting *B-Myb* or by selectively enhanced promoter activity. This dual
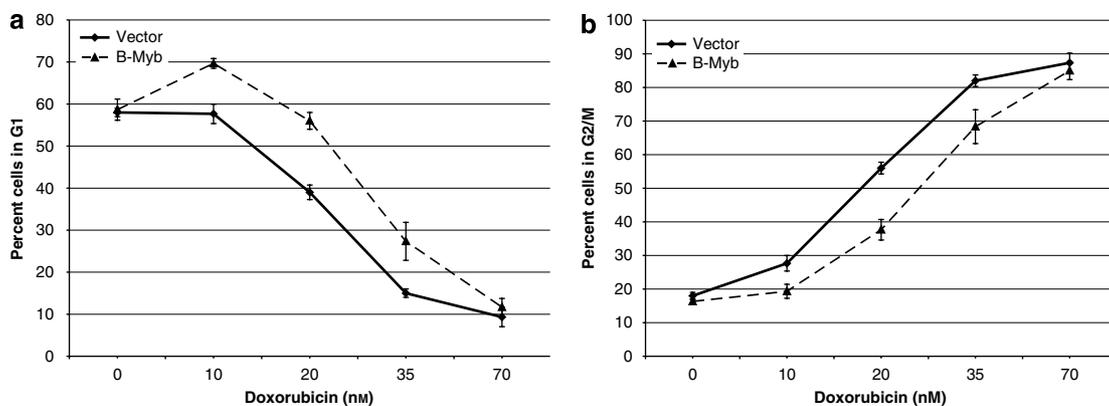

Figure 5 Cell cycle profile of HME-CC cells stably expressing *B-Myb* and treated with doxorubicin. Cell cultures were treated with a range of doses of doxorubicin for 48 h, followed by propidium iodide DNA content analysis. Percentages of cells in (**a**) G1 phase and (**b**) G2/M were calculated by gating based on DNA content. Error bars indicate standard deviations between three independent experiments.

behavior of basal-like tumors, a subtype typical of chemo-responsiveness but with poor patient outcomes, has been described before and termed the 'basal-like tumor paradox' (Carey *et al.*, 2006), and the data presented here suggest that B-Myb might be a key regulator of this complex phenotype.

Recently, Schwab *et al.* (2007) linked the nonsynonymous *B-Myb* germline variant (S427G, rs2070235) to a decrease in overall cancer risk when combining neuroblastomas, chronic myelogenous leukemia and colon cancers into a single dataset and comparing this grouping of cases with non-cancer-bearing controls. While both studies are technically accurate (that is, similar allele frequencies in the control populations; CBCS: homozygous major allele 72.6%, heterozygous 24% and homozygous minor allele 3.4%; Schwab *et al.*, 2007: 72.1, 27.3 and 0.4%, respectively), the association of *B-Myb* rs2070235 genotype and basal-like breast cancer susceptibility differs from the earlier report and has not been described before. Here, in a population-based case–control study, we found that the rs2070235 minor allele was associated with increased risk of basal-like breast cancer but not other subtypes in the CBCS (Table 1). The discrepancy between the Schwab data and what is reported here may be due to differences between breast cancer and the cancers examined in the Schwab paper. Even among breast cancers there is significant heterogeneity across the subtypes in terms of the etiologic role of the *B-Myb* allele, and an analysis of breast cancer without subdivision would have missed the association of rs2070235 with the basal-like breast cancer subtype.

Presently, the function of the S427G variant is unknown. Our *in vitro* studies in breast epithelial cell lines showed neither a phenotypic difference between the overexpression of *B-Myb* or its variant on chemosensitivity relative to each other, nor did we observe a difference in their baseline gene expression patterns. It is temping to speculate that rs2070235 has functional consequences and somehow contributes to the etiology of basal-like tumors. The *B-Myb* variant was found to be nearly 10-fold more frequent in African-Americans (7%) than in non-African-Americans (0.8%) in the CBCS. This is relevant in light of recent data showing that premenopausal African-Americans are approximately twice as likely to develop basal-like tumors compared with premenopausal Caucasians (Carey *et al.*, 2006; Millikan *et al.*, 2008). Of note, Schwab *et al.* (2007) showed that the B-Myb S427G protein was more stable than the wild-type protein. This increased stability may correspond to elevated *B-Myb* protein levels, possibly increasing transcriptional activity of G2/M cell cycle genes and leading to higher inherent proliferation rates. This alteration of cell cycle control may contribute to *B-Myb*'s influence on poor outcome breast cancers. Additional assays are required to completely investigate the role of rs2070235 and other variants in *B-Myb*, which may also lie in linkage disequilibrium with the G allele. This is an important area of investigation, as it is possible that one or more variants in *B-Myb* could contribute to the higher frequency of basal-like breast

cancer among African-American breast cancer patients and also to the pathophysiology of basal-like breast cancer.

As chemotherapy is currently the only option for basal-like cancer patients, we explored whether increased *B-Myb* expression had any effect on chemosensitivity *in vitro*. We observed a statistically significant increase in sensitivity to two TOP2A inhibitors, doxorubicin and etoposide, in the HME-CC and ME16C cells overexpressing *B-Myb* or its variant, but this phenotype was not observed in the basal-like tumor-derived cell lines (Figure 3). Also, there was no significant difference in chemosensitivity to the other tested drugs in any of the cell lines, which included camptothecin, a DNA topoisomerase I (TOP1) inhibitor. TOP2A, a nuclear enzyme that relaxes both negative and positive DNA supercoils by creating double-stranded DNA breaks, is of particular importance for proper DNA duplication during the S phase of the cell cycle (Smith *et al.*, 1994). TOP2A inhibitors cause the enzyme to become trapped on double-strand DNA breakpoints, thereby causing G2 cell cycle checkpoint arrest. As *B-Myb* is a G2/M regulating gene, increased *B-Myb* expression may be promoting the TOP2A-inhibitor-treated cells via the G2 checkpoint through induction of downstream *B-Myb* target genes. By facilitating cells through G2, with less regard for DNA damage, the *B-Myb*-overexpressing cells may attempt to cycle again, eventually leading to the observed increased sensitivity to TOP2A phenotype. TOP2A itself was on the *B-Myb* induced gene list (Supplementary Table 2), and thus more of the target of doxorubicin and etoposide was present, adding to the observed sensitivity phenotype in *B-Myb*-overexpressing cells.

In support of this hypothesis, the gene list identified by microarray analysis as being highly expressed in doxorubicin-treated, *B-Myb*-overexpressing cells (Supplementary Table 2) was significantly enriched in genes required for G2/M progression. For example, this list included the newly described *B-Myb* interacting protein LIN-9, a protein required along with *B-Myb* for the transcription of G2/M genes (Osterloh *et al.*, 2007). In addition, our *B-Myb* target gene list contained many *B-Myb* targets identified earlier, including *CDC2*, *Cyclin B1* and *BIRC5* (Zhu *et al.*, 2004; Osterloh *et al.*, 2007). This gene list represents putative mammary *B-Myb* target genes and again suggests that *B-Myb* has its greatest effect on G2/M genes. In agreement with the gene list enrichment results, when *B-Myb*-overexpressing cells were treated with low doses of doxorubicin or etoposide, more cells accumulated in G1 versus controls, suggesting that the control cell line was appropriately inhibiting cell cycle progression at G2, whereas *B-Myb*-overexpressing lines were bypassing this checkpoint (Figure 5).

The results of this study enhance our understanding of the role of *B-Myb* in breast cancer by identifying new *B-Myb* target genes, by showing that this gene is highly expressed in basal-like breast cancers and that it is of prognostic value for survival and predictive value for pathological complete response. Also, we have described

a significant correlation between a *B-Myb* variant and an increased risk of basal-like breast cancer. These findings point to *B-Myb* as a biomarker that is of potential clinical importance for determining disease risk and for guiding treatment. In addition to its role in basal-like cancers, *B-Myb* may also be of great importance in luminal tumors, a breast cancer subtype with relatively good prognosis, as *B-Myb* expression was capable of stratifying the poor from the good actors within this group, and it is within this group where *B-Myb* is occasionally amplified on the DNA level. The link between *B-Myb* high expression and increased chemotherapy sensitivity *in vitro* is mirrored by similar findings *in vivo* where we and others have shown that basal-like tumors are, on average, the most sensitive to multi-agent chemotherapy regimens that contain an anthracycline (Rouzier *et al.*, 2005; Carey *et al.*, 2007). It is unlikely that *B-Myb* expression alone is responsible for the chemotherapy sensitivity of basal-like tumors, but when coupled with the loss of TP53 function, which is known to occur in basal-like tumors (Sorlie *et al.*, 2001), and the loss of RB function (Derenzini *et al.*, 2008; Herschkowitz *et al.*, 2008), multiple important checkpoints are deficient in basal-like tumors and it is this lack of control that may ultimately prove to be their Achilles' heel.

Materials and methods

Cell lines

hTERT-immortalized, human mammary epithelial cell lines (HME-CC, ME16C) and basal-like breast cancer-derived lines (SUM102, SUM149) were cultured as described (Troester *et al.*, 2004; Hoadley *et al.*, 2007). Full-length, human *B-Myb* complementary DNA (GenBank NM_002466) was cloned into the pBabe.puro.GWrfA (Gateway Reading Frame A) vector using Gateway Cloning Technology (Invitrogen, Carlsbad, CA, USA). Retrovirus was produced in Phoenix 293T cells by transfecting with 10 μg of vector using Lipofectamine 2000 (Invitrogen), as per the manufacturer's instructions. Media was changed 24 h after transfection and supernatants were collected 12 h later. Seventy-five micrograms of polybrene was added to the collected supernatants and applied to the mammary cell lines. Stable populations were selected by culturing in 1 μg/ml puromycin for HME-CC and ME16C, or 0.5 μg/ml puromycin for SUM102 and SUM149.

B-Myb variant (S427G) was created in the pBabe.puro.*B-Myb* expression vector using QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, Los Angeles, CA, USA).

Western blot analysis

Cells were grown in 10-cm tissue culture-treated dishes until 80% confluence, followed by harvest, protein isolation and quantification as described earlier (Troester *et al.*, 2004). Membranes were probed for *B-Myb* (sc-725; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and β-actin (AC-15; Abcam, Cambridge, MA, USA), followed by anti-rabbit or anti-mouse IgG horseradish peroxidase-linked whole antibody (Amersham Biosciences, Piscataway, NJ, USA) and detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA).

Cell cycle analysis

Cells were treated with a range of doxorubicin (0–70 nM) or etoposide (0–2 nM) doses and DNA content was analysed using a modified propidium iodide-staining assay. Briefly, one million cells were collected by trypsinization, washed in $1 \times$ PBS, and fixed in 70% ethanol at 4 °C overnight. Cells were washed with PBS/0.2% BSA and resuspended in 500 μl of PBS/0.2% BSA and 100 μg RNaseA. Propidium iodide was added to a final volume of 50 μg, and cells incubated at 37 °C for 30 min. DNA content analysis was performed using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). To determine fractions of cells in G1 and G2/M phases, histograms of DNA content were analysed using Summit v4.3 software (Dako, Carpinteria, CA, USA) gating around 2 N and 4 N DNA content. Analyses were done on three separate days and mean values were calculated.

Cytotoxicity assay

Cell line sensitivities to drugs were assessed using a modified mitochondrial dye-conversion assay (Cell-Titer 96, Promega #G4100, Madison, WI, USA) as described (Troester *et al.*, 2004; Hoadley *et al.*, 2007). Chemotherapeutics (carboplatin, doxorubicin, 5-fluorouracil, paclitaxel, etoposide, camptothecin) were purchased from Sigma (St Louis, MO, USA). The 72-h inhibitory concentration that caused a 50% reduction in MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) dye conversion (IC50) was determined using nonlinear regression (SAS Statistical Software, Cary, NC, USA) (Vanewijk and Hoekstra, 1993). Differences in the IC50 estimates were tested. A traditional ANOVA test of nested models was performed using the R system for statistical computing (R Development Core Team, 2006, http://www.R-project.org).

Microarray analysis

Five replicates each of HME-CC *B-Myb*-overexpressing and vector control cell lines were treated with the 72-h IC50 dose of doxorubicin. Poly-A (+) RNA was collected (Micro-FastTrack2.0 mRNA Isolation Kit, Invitrogen) from treated control cells and *B-Myb*-overexpressing lines, reverse transcribed and labeled using the Agilent Low RNA Input Linear Amplification Kit (Agilent Technologies, Santa Clara, CA, USA), and hybridized to Agilent Human 44K Custom Oligo microarrays as described (Hu *et al.*, 2005). An untreated HME-CC cell line reference was co-hybridized to all arrays (for example, untreated HME-CC versus doxorubicin-treated empty vector HME-CC). Microarrays were scanned on an Axon Genepix 4000B microarray scanner and analysed using GenePix Pro 5.1 software (Molecular Devices, Sunnyvale, CA, USA). Data were normalized using Lowess normalization on the Cy3 and Cy5 channels. Microarray data are available at the UNC Microarray Database [http://genome.unc.edu] and at the Gene Expression Omnibus (GSE11429).

Microarray statistical analyses

Supervised microarray analysis was performed by selecting genes with an absolute signal intensity of at least 30 units in both dye channels and data present in at least 70% of experimental samples. A two-class, unpaired SAM was performed to identify significant genes associated with *B-Myb* expression with a false discovery rate of <3% (Tusher *et al.*, 2001).

The Netherlands Cancer Institute breast cancer dataset (NKI-295, n = 295) was used for analysis of *B-Myb* expression across breast cancer subtypes (van de Vijver *et al.*, 2002); however, only locally treated tumors (no chemotherapy) were used in survival analyses (n = 165). Association of *B-Myb*

expression states (rank ordered and split into halves: low/high) relative to survival was tested using the Cox–Mantel log-rank test and results visualized using Kaplan–Meier survival plots (WinSTAT v.2007.1). Testing the association of *B-Myb* expression with subtypes was performed using ANOVA. Three other published datasets were analysed, as above, for survival (Miller *et al.*, 2005; Wang *et al.*, 2005) or pathological response (Hess *et al.*, 2006) by χ^2 using the R system for statistical computing.

B-Myb genotyping (CBCS)
The Carolina Breast Cancer Study is a population-based, case–control study of breast cancer conducted in 24 counties of central and eastern North Carolina between 1993 and 2001

(Newman *et al.*, 1995; Millikan *et al.*, 2003). *B-Myb* genotyping (rs2070235) was conducted using DNA extracted from peripheral blood lymphocytes for 1256 cases with subtype information (500 African-American, 756 Caucasian) and 1814 controls (679 African-American, 1135 Caucasian). For complete details see Supplementary Materials.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)