

Y-box binding protein 1 is up-regulated in proliferative breast cancer and its inhibition deregulates the cell cycle

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Abstract. The Y-box-binding protein 1 (YB-1), a member of the cold-shock domain RNA- and DNA-binding protein family, has pleiotropic functions such as regulation of the cell cycle. The aim of this study was to evaluate if YB-1 is a proliferative marker in breast cancer and elucidate potential downstream targets involved in YB-1-mediated cell cycle regulation using RNA interference technology. YB-1 protein expression was evaluated in tissue microarrays of 131 breast invasive ductal carcinomas by immunohistochemistry, while the YB-1 gene expression profile was evaluated in the T-47D, MDA-MB-231, ZR-75-1 and MCF7 breast cancer cell lines. Silencing of the *YB-1* gene in T-47D breast cancer cells was performed using siRNA and the effects of down-regulation of YB-1 on cell growth and regulation of the cell cycle were ascertained. A focused panel of 84 genes involved in cell cycle progression was also examined. In tissue microarrays, YB-1 expression was shown to be associated with proliferating cell nuclear antigen (PCNA) immunostaining. siRNA-mediated silencing of the *YB-1* gene inhibited cell proliferation and induced G1 phase cell cycle arrest in T-47D breast cancer cells. Knockdown of the *YB-1* gene induced up-regulation of two genes which contribute to G1-arrest (*RAD9A* and *CDKN3* genes) and down-regulation of ten genes associated with positive regulation of the cell cycle (*SKP2*, *SUMO1*, *ANAPC4*, *CCNB1*, *CKS2*, *MNAT1*, *CDC20*, *RBBP8*, *KPNA2* and *CCNC* genes). The data obtained from the tissue microarrays and cell lines provide evidence that YB-1 is a reliable marker of cell proliferation and possibly a potential molecular target in breast cancer therapy.

Introduction

Breast cancer is the most frequent malignancy among women in Western countries. It is estimated that more than 1.1 million new breast cancer cases are diagnosed worldwide per year (1). Identification of clinically useful biomarkers is imperative for optimal care and management of breast tumors (2). Several proteins such as estrogen receptors, epidermal growth factor receptors, BRCA1 and progesterone receptors have been reported to be prognostic biomarkers in breast cancer (3,4).

Another potential biomarker which has been investigated in breast cancer is the Y-box binding protein 1 (YB-1) (4,5). YB-1 belongs to a family of DNA- and RNA-binding proteins which participate actively in transcriptional and translational control of cells (5). It is a multifunctional protein involved in a myriad of functions which include gene regulation, DNA repair, drug resistance and cellular response to environmental stimuli (6-8). YB-1 can promote tumor growth and chemoresistance by inducing growth-enhancing genes such as *proliferating cell nuclear antigen (PCNA)*, *DNA topoisomerase IIa*, *thymidine kinase*, *DNA polymerase α* , *EGFR*, *HER-2*, and *multidrug resistance-1 (MDR1/ABCA1)* (9-13).

Increased expression of YB-1 has been reported in a variety of cancers including primary breast cancer (9), prostate cancer (14), primary melanoma (15), colorectal cancer (12) and osteosarcoma (16). Overexpression of YB-1 has been reported in breast cancer carcinomas (9) and 80% of primary breast tumors were reported to show an increased copy number of chromosome 1, the location of *YB-1* (17). Furthermore, YB-1 has also been reported to stimulate proliferation of pre-neoplastic breast cancer cells (18) and phosphorylation of the protein at Ser102 in its cold shock domain by Akt has been observed to influence tumor cell growth (19). It has also been shown that YB-1 can bind EGFR and transcriptionally induce the expression of EGFR (20). Recent studies showed that expression of YB-1 is involved in Twist-promoted tumor cell growth (21) and tumor growth is suppressed by targeting YB-1 in nude mice (22). These previous findings suggest that YB-1 could possibly be a biomarker for proliferation in breast cancer.

Thus, in this study we analyzed the association of YB-1 expression with PCNA immunostaining in breast cancer

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tissue samples by immunohistochemistry and showed that increased YB-1 expression is associated with cell proliferation. We also observed that siRNA-mediated down-regulation of YB-1 expression altered cell cycle progression and the profile of cell cycle-related genes in breast cancer cells *in vitro*.

Materials and methods

Clinical samples. Tissue microarrays comprising 131 invasive ductal breast cancer specimens were obtained from the Singapore General Hospital between 1998 and 2002. Patients received primary surgical treatment (either mastectomy or breast conserving surgery). Ethics approval was obtained from the Institutional Review Board, Singapore General Hospital.

Immunohistochemistry. Tissue microarray sections were deparaffinized, rehydrated and incubated with 3% hydrogen peroxide for 15 min to quench endogenous peroxidase. Antigen retrieval was performed by heating sections in 10 mM citrate buffer (pH 6.0) for 15 min. After rinsing thrice with phosphate-buffered saline-Triton X (PBS-TX), sections were blocked with 5% normal goat serum for 1 h at room temperature. The tissue sections were then incubated with rabbit polyclonal anti-YB-1 antibody (1:250 dilution) and mouse monoclonal anti-PCNA antibody (Sigma-Aldrich, St. Louis, MO; 1:200 dilution) at 4°C overnight, respectively. The anti-YB-1 antibody was raised in a rabbit by injecting a synthetic peptide K13 containing a sequence which corresponds to the N-terminus of human YB-1 protein (23). After washing with PBS-TX, biotinylated secondary antibody (1:200 dilution) was added and incubated for 1 h at room temperature. The staining was visualized by the avidin-biotin-complex technique (ABC Kit, Vector Laboratories, Burlingame, CA), using diaminobenzidine (DAB) as the substrate. The sections were counterstained with hematoxylin. The intensity of cytoplasmic YB-1 staining was scored as 0 (no detectable staining), 1+ (light staining), 2+ (moderate staining) and 3+ (strong staining). The immunoreactivity score (IRS) was computed by multiplying the different staining intensities with the percentage of each staining intensity. Immunostaining for PCNA was assessed by counting the percentage of cells with positive nuclear PCNA staining. Negative control sections omitting the primary antibody were included.

Cell culture. T-47D breast cancer cells, MDA-MB-231 breast cancer cells, ZR-75-1 breast cancer cells, and MCF7 breast cancer cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and maintained in Dulbecco's modified Eagle's medium or RPMI medium containing 10% fetal bovine serum (HyClone Laboratories, Logan, UT) in a 37°C incubator with 5% CO₂.

Transfection with YB-1 siRNA. 5.0x10⁴ cells (grown in 24-well culture dish) or 2.5x10⁵ cells (grown in 6-well culture dish) were incubated in complete culture medium without antibiotics. After 24 h, cells were transfected using Oligofectamine™ Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Two YB-1-specific siRNA duplexes, siYB-1A and siYB-2A were obtained from Ambion (Austin,

TX). The siYB-1A and siYB-2A sequences were sense 5'-GCA AAA UUA CCA GAA UAG Utt-3', antisense 5'-ACU AUU CUG GUA AUU UUG Ctg-3' and sense 5'-GCA GAC CGU AAC CAU UAU At 3', antisense 5' UAU AAU GGU UAC GGU CUG Ctg-3' respectively. After 8 h incubation, growth medium containing 30% serum was added and the medium was changed the following day. Subsequently, transfected cells were incubated in medium with 10% FBS for 48 or 72 h for further analysis.

Quantitative real-time RT-PCR. Total RNA of harvested cells was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany) and reverse transcribed to cDNA using SuperScript III 1st-Strand Synthesis System (Invitrogen) following manufacturer's protocols. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA expression level was used as a reference. *YB-1* and *GAPDH* transcript levels were determined using QuantiTect SYBR Green Master Mix (Qiagen). Gene specific primers used for *YB-1* and *GAPDH* were 5'-AAG TGA TGG AGG GTG CTG AC-3' for YB-1 forward primer, 5'-TTC TTC ATT GCC GTC CTC TC-3' for YB-1 reverse primer; and 5'-GAA GGT GAA GGT CGG AGT CAA CG-3' for *GAPDH* forward primer and 5'-TGC CAT GGG TGG AAT CAT ATT GG-3' for *GAPDH* reverse primer. The cycling parameters were performed as indicated: initial denaturation at 95°C for 15 min, followed by 45 cycles of denaturation at 94°C for 15 sec, annealing at 60°C for 25 sec, and elongation at 72°C for 12 sec. The specificities of YB-1 and *GAPDH* primers were confirmed by melting curve analysis and also electrophoresis of PCR products on a 2% agarose gel. Relative quantification was calculated using $\Delta\Delta C_T$ and $2^{-\Delta\Delta C_T}$ method, where ΔC_T refers the difference between the C_T values of the target gene and the housekeeping gene *GAPDH*.

Western blot analysis. Twenty micrograms of extracted proteins were separated by SDS-PAGE and transferred onto PVDF membrane. The primary antibodies used were rabbit polyclonal YB-1 (1:1000 dilution), rabbit polyclonal CDKN3 (1:1000 dilution, Abcam, Cambridge, UK), rabbit polyclonal RAD9A (1:100 dilution, Abcam) and mouse monoclonal β -actin (1:6000, Sigma-Aldrich). Specific binding with the appropriate secondary antibodies was detected by enhanced chemiluminescence (Amersham, Little Chalfont, UK). The relative protein expression level was determined by densitometry measurement of the band intensity and normalization with β -actin.

Cell proliferation assay. Cell proliferation was performed in a 24-well plate and conducted with MTS CellTiter 96® AQueous Non Radioactive Cell Proliferation Assay (Promega, Madison, WI). MTS/PMS solution (100 μ l) was added to the siRNA transfected cells in a 24-well plate containing 500 μ l of fresh culture medium. The plate was then incubated for 4 h at 37°C in a humidified, 5% CO₂ atmosphere. The absorbance was read at 490 nm using an ELISA plate reader.

Growth curve analysis. The growth curve was obtained using alamarBlue (Invitrogen). Cells were seeded in 6-well plates and

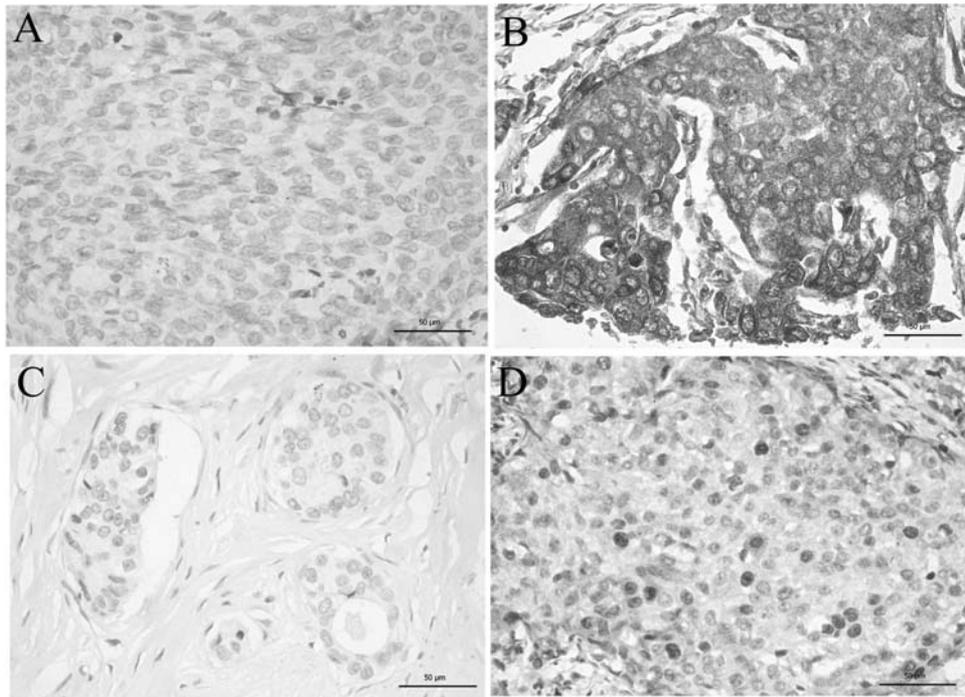


Figure 1. Immunohistochemical expression of YB-1 and PCNA in breast cancer. (A) Non-detectable YB-1 immunostaining in breast cancer tissue section. (B) Strong YB-1 (3+) immunostaining in breast cancer tissue section. (C) Negative PCNA staining in breast cancer tissue section. (D) Positive nuclear PCNA staining in breast cancer tissue section. Hematoxylin counterstain. Scale bars, 50 μ m.

transfected with siRNA. After 20 h, medium was removed and the wells were washed with PBS. AlamarBlue (1:10) in complete culture medium was added to each well and the plates were incubated for 4 h under normal growth conditions before the 24, 48, 72 and 96 h time-points. The absorbance readings were obtained at 570 nm wavelength with 600 nm as the reference wavelength.

Cell cycle analysis. Cell cycle assay was performed in triplicates and repeated thrice. Both floating and adherent cells were collected. Collected cells were fixed with 1 ml of 70% ice-cold ethanol for at least 1 h at 4°C. After washing twice with PBS, the cells were suspended in propidium iodide/RNase A solution and incubated for 30 min at room temperature before analysis.

Superarray analysis. The Human Cell Cycle RT² Profiler™ PCR Array (Superarray Bioscience, Frederick, MD) was utilized to examine the expression of various genes involved in cell cycle progression using cDNA of siNegative treated cells as the negative control at 48 h post transfection. Real-time PCR was performed using Superarray RT² qPCR Master Mix according to the manufacturer's protocol. The experiments were performed in triplicates. The mean C_T value for each gene in the siYB-1 treated versus siNegative treated group was determined and used to calculate the fold-change.

Statistical analysis. The SPSS software Version 12.0 for Windows was used for statistical analysis. Immunostaining of YB-1 was correlated with PCNA staining using Fisher's exact test. A two-tailed Student's t-test was performed for analysis of the statistical significance between two variables and the one-way ANOVA with *post hoc* test used for analysis

Table I. Correlation of YB-1 immunostaining with PCNA positivity.

	YB-1 staining IRS \leq 60	YB-1 staining IRS >60	p-value
PCNA staining			
Negative staining	11	2	0.017
Positive staining	56	62	

of three or more variables. A two-way ANOVA was used to compare statistical differences between growth curves. $p < 0.05$ was considered as statistically significant.

Results

YB-1 expression correlates with cell proliferation in breast cancer tissues. The YB-1 protein was observed to be undetectable in 15 (11.5%) patient samples and expressed in tumor cells from 116 (88.5%) breast cancer patients (Fig. 1A and B). For the positive cases, YB-1 was found to be expressed in the cytoplasm of all tumor cells with absent or faint nuclear staining (Fig. 1B). PCNA nuclear immunostaining was absent in 13 (9.9%) cases and positive in 118 cases (90.1%) (Fig. 1C and D). For analyzing the correlation with PCNA staining, YB-1 immunostained samples with IRS >60 were considered as having high YB-1 expression and IRS \leq 60 as having low YB-1 expression. It was observed that breast cancer specimens with positive PCNA staining had a higher YB-1 expression compared to those with negative PCNA staining ($p=0.017$) (Table I).

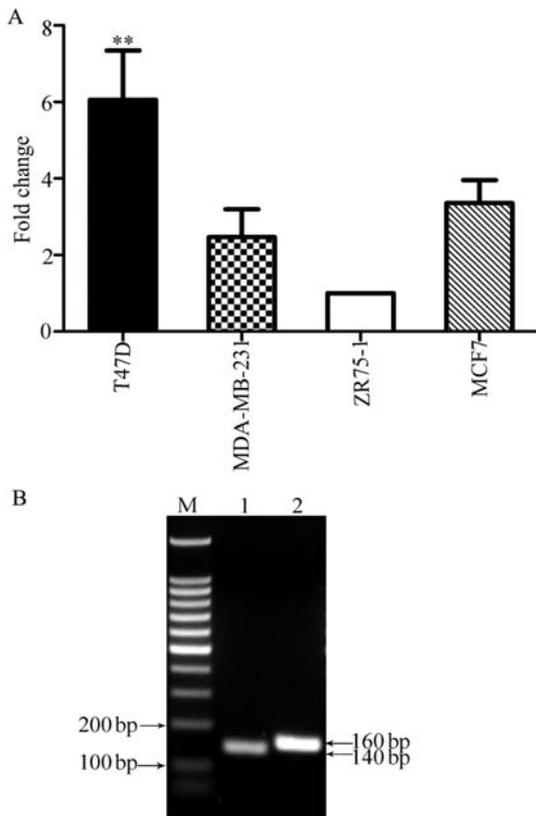


Figure 2. Differential expression of YB-1 in breast cancer cell lines. (A) Relative expression of YB-1 mRNA levels as determined by quantitative real-time RT-PCR in T47D, MDA-MB231, MCF7 and ZR-75-1 breast cancer cell lines. ZR75-1 breast cancer cell line was designated as the reference cell line and *GAPDH* was used for normalization. Values are means of triplicates. Error bar, SEM. ** $p \leq 0.001$. (B) Representative of gel electrophoresis of RT-PCR products of *YB-1* and housekeeping gene *GAPDH*. Lane designation: lane M, molecular weight marker; lane 1, *YB-1*; lane 2, *GAPDH*.

YB-1 mRNA is expressed in breast cancer cell lines. To further evaluate the role of YB-1 in cell proliferation, expression of *YB-1* mRNA was first determined in breast cancer cell lines. As shown in Fig. 2A, *YB-1* was found to be constitutively expressed in all the breast cell lines investigated. Using ZR-75-1 cells as the calibrator cell line, T-47D cells were found to have the highest *YB-1* expression ($p < 0.05$). Hence, T-47D breast cancer cells were selected for silencing of the *YB-1* gene with siRNA for further functional studies. The specificities of *YB-1* and *GAPDH* primers were verified by melting curve analyses (not shown) and running the amplicons on a 2% gel (Fig. 2B).

Silencing of YB-1 gene in T-47D breast cancer cells reduces YB-1 mRNA and protein expression. The knockdown efficiency of siYB-1A and siYB-1B at 48 h in T-47D cells were 87% and 81%, respectively (Fig. 3A). Western blot analysis revealed a significant reduction in YB-1 protein expression level in siYB-1 treated cells compared with siNegative treated cells (Fig. 3B). There was a 36% (siYB-1A) and 48% (siYB-1B) reduction of the YB-1 protein in siYB-1 treated cells.

Silencing of YB-1 gene in T-47D breast cancer cells inhibits cell proliferation and deregulates cell cycle. siYB-1A and

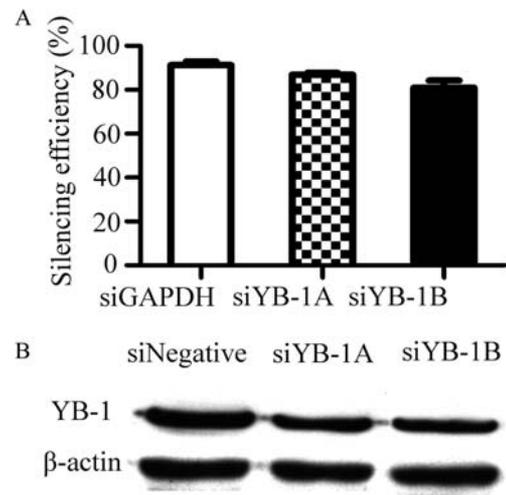


Figure 3. Silencing the *YB-1* gene down-regulates YB-1 expression at mRNA and protein levels. (A) Silencing efficiency of *YB-1* by siYB-1A and siYB-1B as analyzed by real-time RT-PCR in T-47D breast cancer line. siGAPDH was used as a positive control to optimize transfection conditions. (B) Down-regulation of YB-1 protein in T-47D cells as shown by Western blotting after silencing of the *YB-1* gene. β -actin was used as a normalizer. siYB-1 treatment resulted in 35-50% protein down-regulation.

siYB-1B treatment induced a decrease in cell proliferation at 48 h post-transfection (Fig. 4A; $p = 0.050$ and $p = 0.047$, respectively). This result was mirrored by the growth curve analysis which showed a significant decline in cell growth in siYB-1A and siYB-1B treated cells compared with siNegative treated cells (Fig. 4B; $p = 0.043$ and 0.024 , respectively). There was no significant difference in growth in siYB-1A versus siYB-1B treated cells ($p = 0.428$). Cell cycle analysis revealed that there was an increase in the number of cells in G1 phase and decrease in the S and G2/M phases in T-47D cells treated by both *YB-1* specific siRNA duplexes at 48 h and 72 h post-transfection (Fig. 4C and D). At 48 h post-transfection, *YB-1* siRNA treatment induced significant increase in G1 cell fraction as compared to negative control siRNA treated cells, (siYB-1A: 75.8 vs. 63.6%, $p = 0.0004$; siYB-1B: 76.4 vs. 63.6%, $p = 0.0003$). This increase was accompanied by a concomitant reduction in the S phase (siYB-1A: 9.0 vs. 11.5%, $p = 0.004$; siYB-1B: 8.6 vs. 11.5%, $p = 0.006$) and G2/M phase cell fraction (siYB-1A: 15.2 vs. 24.5%, $p = 0.001$; siYB-1B: 14.9 vs. 24.5%, $p = 0.001$). Similar alterations in the phases of the cell cycle were also observed in T-47D cells 72 h after transfection.

Silencing the YB-1 gene modulates expression of cell cycle-related genes. To have a better insight into the role of YB-1 on cell proliferation, the expression profile of a focused panel of 84 cell cycle related genes known to either positively or negatively regulate the cell cycle was analyzed. Expression of 12 genes was found to be significantly altered after 48 h treatment with siYB-1 (Table II and Fig. 5A). Two genes, *cyclin-dependent kinase inhibitor 3 (CDKN3)* and *RAD9 homolog A (RAD9A)*, were up-regulated by 1.62- and 2.60-fold, respectively while the other 10 genes were down-regulated in response to siYB-1 treatment (*SKP2*: 1.33-fold; *SUMO1*, 1.11-fold; *ANAPC4*, 1.46-fold; *CCNB1*, 1.35-fold; *CKS2*,

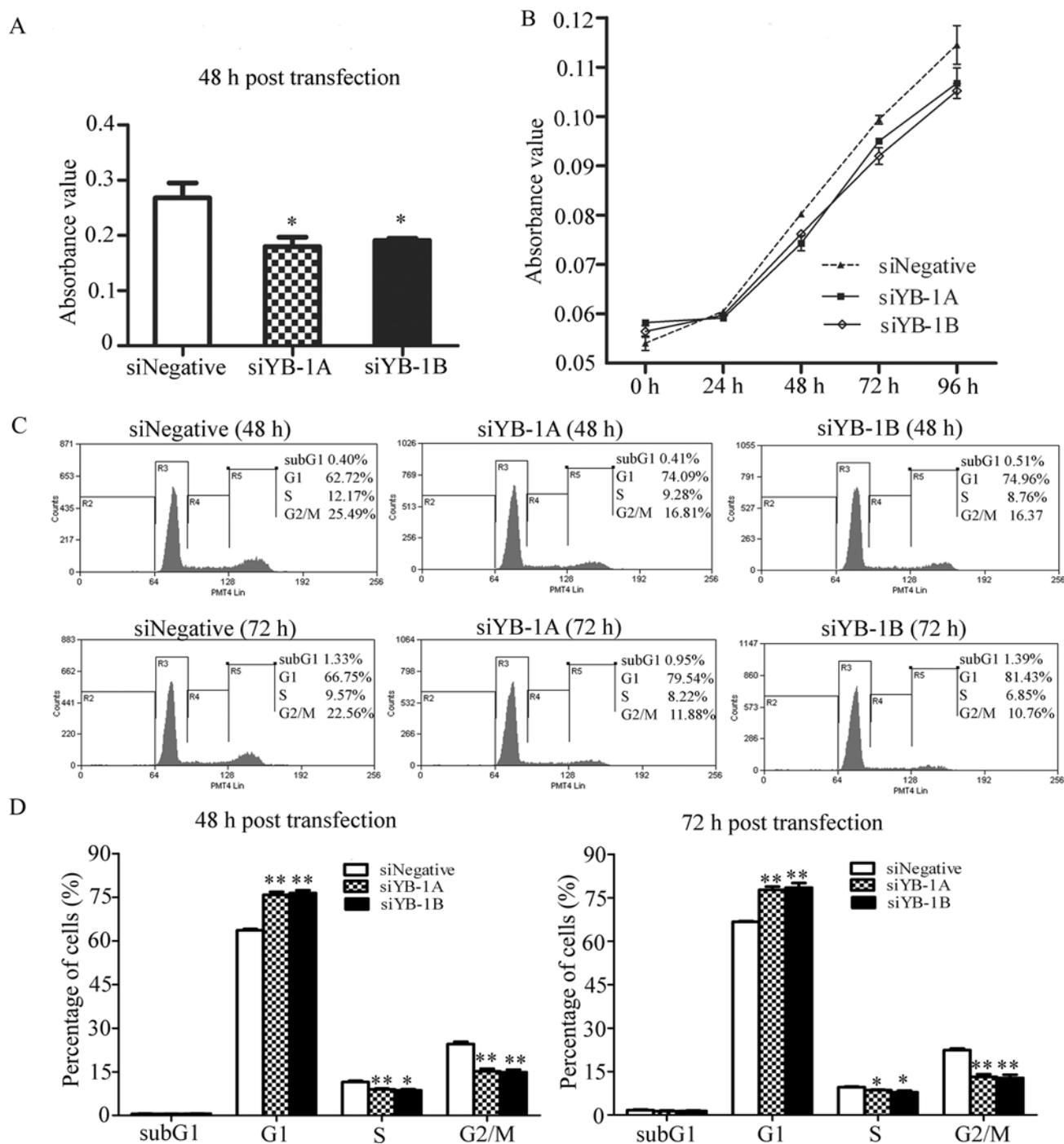


Figure 4. Silencing the *YB-1* gene reduces proliferation and alters cell cycle progression in T-47D breast cancer cells. (A) Cell proliferation assay was performed in T-47D cells at 48 h post-transfection with either siNegative or siYB-1. (B) Growth curve of T-47D cells transfected with siNegative or siYB-1 monitored up to 96 h post-transfection. (C) Representative cell cycle profile of T-47D cells transfected with siNegative, siYB-1A or siYB-1 analyzed by flow cytometry at 48 and 72 h post-transfection. (D) Cell fractions in the different cell cycle phases for siNegative, siYB-1A or siYB-1B treated T-47D cells at 48 and 72 h post-transfection, respectively. Values are means of triplicates. Error bar, SEM. * $p \leq 0.05$, ** $p \leq 0.001$.

1.28-fold; *KPNA2*, 1.14-fold; *MNAT1*, 1.84-fold; *CDC20*, 1.38-fold; *RBBP8*, 1.43-fold and *CCNC*, 1.18-fold). Differential protein expression levels of *CDKN3* and *RAD9A* were also verified by Western blotting as shown in Fig. 5B.

Discussion

YB-1 has been reported to be overexpressed in about 75% of breast cancer carcinoma and associated with poor survival

and high rates of relapse in almost all breast cancer subtypes (4,9). *YB-1* also possesses growth-enhancing potential in various cancers by promoting cell proliferation through transcriptional regulation of relevant genes. Although two main regulators of cell cycle progression, cyclin A and cyclin B1, have been found to be associated with *YB-1* (18), other potential key cell cycle-related genes mediated by *YB-1* which affects breast cancer progression have not been determined.

Table II. Focused panel of the cell cycle related genes analyzed.

Gene	Description	Gene	Description
<i>ABL1</i>	C-abl oncogene 1, receptor tyrosine kinase	<i>CKS1B</i>	CDC28 protein kinase regulatory subunit 1B
<i>ANAPC2</i>	Anaphase promoting complex subunit 2	<i>CKS2^b</i>	CDC28 protein kinase regulatory subunit 2
<i>ANAPC4^b</i>	Anaphase promoting complex subunit 4	<i>CUL1</i>	Cullin 1
<i>DIRAS3</i>	DIRAS family, GTP-binding RAS-like 3	<i>CUL2</i>	Cullin 2
<i>ATM</i>	Ataxia telangiectasia mutated	<i>CUL3</i>	Cullin 3
<i>ATR</i>	Ataxia telangiectasia and Rad3 related	<i>DDX11</i>	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 11 (CHL1-like helicase homolog, <i>S. cerevisiae</i>)
<i>BAX</i>	BCL2-associated X protein	<i>DNM2</i>	Dynamamin 2
<i>BCCIP</i>	BRCA2 and CDKN1A interacting protein	<i>E2F4</i>	E2F transcription factor 4, p107/p130-binding
<i>BCL2</i>	B-cell CLL/lymphoma 2	<i>GADD45A</i>	Growth arrest and DNA-damage-inducible, alpha
<i>BIRC5</i>	Baculoviral IAP repeat-containing 5 (survivin)	<i>GTF2H1</i>	General transcription factor IIIH, polypeptide 1 62 kDa
<i>BRCA1</i>	Breast cancer 1, early onset	<i>GTSE1</i>	G-2 and S-phase expressed 1
<i>BRCA2</i>	Breast cancer 2, early onset	<i>HERC5</i>	Hect domain and RLD 5
<i>CCNB1^b</i>	Cyclin B1	<i>HUS1</i>	HUS1 checkpoint homolog (<i>S. pombe</i>)
<i>CCNB2</i>	Cyclin B2	<i>KNTC1</i>	Kinetochore associated 1
<i>CCNC^b</i>	Cyclin C	<i>KPNA2^b</i>	Karyopherin alpha 2 (RAG cohort 1, importin alpha 1)
<i>CCND1</i>	Cyclin D1	<i>MAD2L1</i>	MAD2 mitotic arrest deficient-like 1 (yeast)
<i>CCND2</i>	Cyclin D2	<i>MAD2L2</i>	MAD2 mitotic arrest deficient-like 2 (yeast)
<i>CCNE1</i>	Cyclin E1	<i>MCM2</i>	Minichromosome maintenance complex component 2
<i>CCNF</i>	Cyclin F	<i>MCM3</i>	Minichromosome maintenance complex component 3
<i>CCNG1</i>	Cyclin G1	<i>MCM4</i>	Minichromosome maintenance complex component 4
<i>CCNG2</i>	Cyclin G2	<i>MCM5</i>	Minichromosome maintenance complex component 5
<i>CCNH</i>	Cyclin H	<i>MKI67</i>	Antigen identified by monoclonal antibody Ki-67
<i>CCNT1</i>	Cyclin T1	<i>MNAT1^b</i>	Menage a trois homolog 1, cyclin H assembly factor (<i>Xenopus laevis</i>)
<i>CCNT2</i>	Cyclin T2	<i>MRE11A</i>	MRE11 meiotic recombination 11 homolog A (<i>S. cerevisiae</i>)
<i>CDC16</i>	Cell division cycle 16 homolog (<i>S. cerevisiae</i>)	<i>NBN</i>	Nibrin
<i>CDC2</i>	Cell division cycle 2, G1→S and G2→M	<i>PCNA</i>	Proliferating cell nuclear antigen
<i>CDC20^b</i>	Cell division cycle 20 homolog (<i>S. cerevisiae</i>)	<i>RAD1</i>	RAD1 homolog (<i>S. pombe</i>)
<i>CDC34</i>	Cell division cycle 34 homolog (<i>S. cerevisiae</i>)	<i>RAD17</i>	RAD17 homolog (<i>S. pombe</i>)
<i>CDK2</i>	Cyclin-dependent kinase 2	<i>RAD51</i>	RAD51 homolog (RecA homolog, <i>E. coli</i>) (<i>S. cerevisiae</i>)
<i>CDK4</i>	Cyclin-dependent kinase 4	<i>RAD9A</i>	RAD9 homolog A (<i>S. pombe</i>)

Table II. Continued.

Gene	Description	Gene	Description
<i>CDK5R1</i>	Cyclin-dependent kinase 5, regulatory subunit 1 (p35)	<i>RBI</i>	Retinoblastoma 1 (including osteosarcoma)
<i>CDK5RAP1</i>	CDK5 regulatory subunit associated protein 1	<i>RBBP8^b</i>	Retinoblastoma binding protein 8
<i>CDK6</i>	Cyclin-dependent kinase 6	<i>RBL1</i>	Retinoblastoma-like 1 (p107)
<i>CDK7</i>	Cyclin-dependent kinase 7	<i>RBL2</i>	Retinoblastoma-like 2 (p130)
<i>CDK8</i>	Cyclin-dependent kinase 8	<i>RPA3</i>	Replication protein A3, 14 kDa
<i>CDKN1A</i>	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	<i>SERTAD1</i>	SERTA domain containing 1
<i>CDKN1B</i>	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	<i>SKP2^b</i>	S-phase kinase-associated protein 2 (p45)
<i>CDKN2A</i>	Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	<i>SUMO1^b</i>	SMT3 suppressor of mif two 3 homolog 1 (<i>S. cerevisiae</i>)
<i>CDKN2B</i>	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	<i>TFDP1</i>	Transcription factor Dp-1
<i>CDKN3^a</i>	Cyclin-dependent kinase inhibitor 3 (CDK2-associated dual specificity phosphatase)	<i>TFDP2</i>	Transcription factor Dp-2 (E2F dimerization partner 2)
<i>CHEK1</i>	CHK1 checkpoint homolog (<i>S. pombe</i>)	<i>TP53</i>	Tumor protein p53
<i>CHEK2</i>	CHK2 checkpoint homolog (<i>S. pombe</i>)	<i>UBA1</i>	Ubiquitin-like modifier activating enzyme 1

^aGene up-regulated; ^bgene down-regulated.

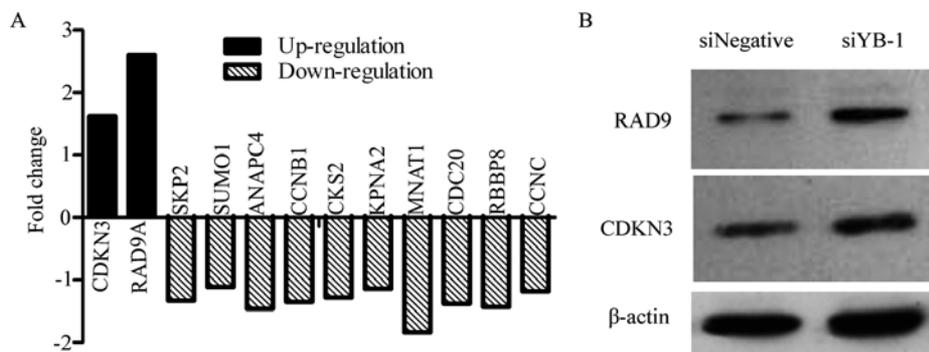


Figure 5. Silencing the *YB-1* gene in T-47D breast cancer cells modulates cell cycle-related genes. (A) Genes differentially expressed by silencing the *YB-1* gene ($p < 0.05$). Values are obtained based on C_T values of triplicates. (B) Silencing of *YB-1* gene induced up-regulation of CDKN3 and RAD9A proteins as analyzed by Western blotting. Representative blots of triplicate experiments.

The primary focus of this study was to identify a prognostic marker of cell proliferation and a promising molecular target in breast cancer therapy. In the present study, immunopositive staining of YB-1 in tissue microarray specimens was observed to be significantly associated with PCNA, an established marker of cell proliferation. The relationship between YB-1 expression and cell proliferation in tumor samples is substantiated by *in vitro* evidence where siRNA-mediated knock-down of the *YB-1* gene in T-47D breast cancer cells (which had the highest level of YB-1 expression of the four

breast cancer cell lines examined) resulted in a significant decrease in cell growth compared to siNegative treated cells. Cell cycle analysis in T-47 D cells and MDA-MB231 cells revealed a significantly increased G1 cell fraction which was accompanied by a decrease in the S phase and G2/M phase in siYB-1 treated cells compared to siNegative treated cells. To further investigate the underlying molecular basis that may contribute to observed alterations in the cell cycle, a Superarray analysis of 84 cell cycle-related genes was carried out in T-47D breast cancer cells. We identified the

following genes that were modulated in YB-1-regulated cell cycle progression, *viz.*, *CDKN3*, *RAD9A*, *SKP2*, *SUMO1*, *ANAPC4*, *CCNB1*, *CKS2*, *MNAT1*, *CDC20*, *RBBP8*, *KPNA2* and *CCNC*. Among them, *CDKN3* and *RAD9A* were found to be up-regulated at mRNA and protein levels involved in G1-arrest, while the other 10 cell cycle genes were down-regulated.

Cell cycle progression is mainly controlled by activation of cyclin-dependent kinases (CDKs) which regulates transition from one cell cycle phase to the next phase. *CDKN3* encodes a kinase-associated phosphatase (KAP) which is an important regulator of cell cycle progression (25,26). KAP selectively inhibits Cdk2 by dephosphorylating a threonine residue (Thr160), thereby reducing its ability to phosphorylate the retinoblastoma protein (pRb). Non-phosphorylated pRb binds transcription factor E2F1 and prevents generation of proteins required for G1/S transition (26-28). Hence, decreased cell proliferation observed with knockdown of YB-1 could be due in part to up-regulation of *CDKN3*. This is supported by the finding that mouse embryo cells with YB-1 deficiency express accumulated levels of the G1-specific CDK-inhibitors p16^{INK4a} and p21^{CIP} and senesce prematurely (29).

Besides *CDKN3*, our results also show that YB-1 can control G1/S transition by regulating the expression of the *RAD9A* gene and protein. *RAD9A* (also known as *RAD9*) is evolutionarily conserved (30) and the encoded protein plays key roles in several fundamental biological processes including mediating the cell cycle (31,32), acting as a pro-apoptotic element (33) and maintaining genomic integrity (34). It has been documented that *RAD9A* can bind *RAD1* and *HUS1*, forming a checkpoint protein complex which is capable of recognizing DNA damage and delay cell cycle progression (35,36). Like p53, *RAD9A* has been found to have the capability of controlling the progression of cells from G1 to S by activating transcription of *p21* gene (37), which encodes a potent cyclin-dependent kinase inhibitor (CKI), leading to G1-arrest. Beyond this, *RAD9A* is also involved in cell-cycle arrest in G2/M phase and prevents entry into mitosis by inhibiting the nuclear division pathway which requires *Cdc20* function (38), resulting in reduced cell growth (39). *Cdc20* activates the anaphase-promoting complex/cyclosome in mitosis which facilitates chromosome segregation (40). Taken together, si-YB1 mediated reduced cell growth could also be partially explained by up-regulation of *RAD9A* and concurrent down-regulation of *Cdc20*.

Our finding that the *CCNB1* gene, which controls G2-M transition of the cell cycle by complexing with *CDK1* (41), was inhibited by knockdown of YB-1 is in agreement with the previous finding that as a cell cycle stage-specific transcription factor, YB-1 facilitates *cyclin B1* gene transcription (18). *ANAPC4* drives metaphase-anaphase transition by ubiquitinating its specific substrates such as mitotic cyclins and anaphase inhibitor and facilitates cell cycle progression. *CKS2* proteins are essential components of cyclin/cyclin-dependent kinase complexes involved in cell cycle regulation (42). Another down-regulated protein *MNAT1* is involved in assembly of CDK-activating kinase (CAK) complex which phosphorylates and activates essential cell cycle regulators - CDKs (43). The S-phase kinase-associated protein *Skp2* is required for the ubiquitin-mediated degradation of the Cdk

inhibitor p27 which arrests cell cycle in G1 phase (44,45). *RBBP8* encodes CtIP, a phosphorylation-dependent binding partner of BRCA1 C-terminal domains and BRCA1/CtIP complex is required for G2/M transition induced by DNA damage (46). The other three genes, including *SUMO1*, *KPNA2* and *CCNC*, may not be biologically significant due to the slight fold changes (<1.20).

Our present study has revealed that cell cycle-related genes regulated by YB-1 are involved in tumor growth, which is consistent with previous findings that down-regulation of YB-1 expression induced growth inhibitory effects associated with increased apoptosis (18,21,22,47), although the underlying mechanisms governing YB-1-mediated cell growth are still not fully understood. It has been reported that YB-1 can be phosphorylated by Akt and promote tumor cell growth through Akt signal transduction pathway (19) and that Twist up-regulates *AKT2* (48) and induces tumor growth regulation of YB-1 (21). Lately, another G1/S phase protein, *CDC6* which is essential for the initiation of DNA replication was also found to be modulated by YB-1 (49). However, factors influencing tumor growth may be more complex than it seems. Recently, it was reported that enforced YB-1 expression in H-ras transformed MCF 10AT breast epithelial cells promoted epithelial-mesenchymal transition accompanied by increased metastatic potential with reduced proliferation rates (50).

In conclusion, we have demonstrated that there is a significant association between YB-1 and cell proliferation in both breast cancer tissues and *in vitro*. Moreover, cell cycle analysis revealed that YB-1 regulates cell cycle progression by modulating expression of 12 cell cycle-related genes. It would seem that regulation of the cell cycle by YB-1 requires a complex interplay of cell cycle related genes. YB-1 is a potentially clinically useful biomarker of proliferation in breast cancer and a promising molecular target for further development as a novel agent in breast cancer therapy.

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