Genes to Cells

YB-1 promotes transcription of *cyclin D1* in human non-small-cell lung cancers

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Cyclin D1, an oncogenic G1 cyclin, and YB-1, a transcription factor involved in cell growth, are both over-expressed in several human cancers. In human lung cancer, the functional association between YB-1 and cyclin D1 has never been elucidated. In this study, we show YB-1 is involved in the transcription of *cyclin D1* in human lung cancer. Depletion of endogenous YB-1 by siRNA inhibited progression of G1 phase and down-regulated both the protein and mRNA levels of cyclin D1 in human lung cancer cells. Forced over-expression of YB-1 with a cyclin D1 reporter plasmid increased luciferase activity, and ChIP assay results showed YB-1 bound to the *cyclin D1* promoter. Moreover, the amount of *YB-1* mRNA positively correlated with *cyclin D1* mRNA levels in clinical non-small-cell lung cancer (NSCLC) specimens. Immunohistochemical analysis also indicated YB-1 expression correlated with cyclin D1 expression in NSCLC specimens. In addition, most of the cases expressing both cyclin D1 and CDC6, another molecule controlled by YB-1, had co-existing YB-1 over-expression. Together, our results suggest that aberrant expression of both cyclin D1 and CDC6 by YB-1 over-expression may collaboratively participate in lung carcinogenesis.

Introduction

Lung cancer is the leading cause of cancer death worldwide, including in Japan (Jemal *et al.* 2011). In particular, non-small-cell lung cancer (NSCLC) accounts for 85% of human lung cancers. Despite significant progress in NSCLC treatment, such as chemotherapy, radiotherapy, and surgery, the prognosis for patients with NSCLC has been improved only minimally and the 5-year survival rate remains at 15% (Molina *et al.* 2008). Recent advances in the molecular characterization of NSCLC have enabled the identification of numerous cell growth and proliferation pathways that are disrupted in these tumors. These findings have provided insights into the mechanisms of tumor development in various histologic subtypes of NSCLC and have pointed toward targeted treatment strategies (Sanders & Albitar 2010). Li Ding *et al.* identified several mutated genes in NSCLC, including tumor suppressor and tyrosine kinase genes that may function as proto-oncogenes. Furthermore, the authors found a significant excess of mutations and copy number alterations in genes from

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the MAPK, p53, Wnt, cell cycle, and PI3K mTOR signaling pathways, suggesting that these pathways are linked to the disease development and progression (Okudela *et al.* 2007; Ding *et al.* 2008). Many of the genes and proteins involved in lung cancer pathogenesis can be categorized into three major pathways: cell cycle regulation, apoptosis, and angiogenesis. The molecules in these three pathways have been also investigated from the standpoint of their influence on the clinical outcome of NSCLC (Singhal *et al.* 2005).

Cyclins and their associated cyclin-dependent kinases (CDK) are the central machinery that control cell cycle progression. In G1 phase, once the Rb protein is phosphorylated by the cyclin D1/CDK complex, E2F is released, allowing transcription of other CDKs, cyclins, and S phase proteins, thereby promoting the transition from G1 to S phase of the cell cycle. During S phase, cyclin D1 is phosphorylated by glycogen synthase kinase 3β, which promotes nuclear export and ubiquitination of cyclin D1 by the SCF^{Fbox4}–αB crystalline complex, leading to proteasome-dependent degradation of cyclin D1. Other F-box proteins, such as Fbxw8, Fbox31, and Skp2, have also been reported as E3 ligases for cyclin D1, but knockout mouse analyses of these genes indicated that the contribution of these reported E3s toward degradation of cyclin D1 were not significant (Kanie *et al.* 2012). Therefore, rather than the ubiquitin proteasome-mediated degradation system, transcriptional regulation may be more important in controlling cyclin D1 expression.

Induction of cyclin D1 in the G1 phase depends on several growth factors, such as EGF and IGF, and several hormones, including estrogen (17β-estradiol:E2) and angiotensin II, until the restriction point (Klein & Assoian 2008; Witzel *et al.* 2010). Until the point of irreversible transition from G1 to S phase, cyclin D1 expression is tightly regulated at the level of transcriptional activation. Several transcriptional factors, including TCF/LEF, CREB, NF-κB, AP-1, and SP1, have been found to transactivate the *cyclin D1* promoter, and some transcriptional suppressors, such as Tob1 and Jumonji, have been reported to down-regulate *cyclin D1* gene promoter activity (Guttridge *et al.* 1999; Lee *et al.* 1999; Shtutman *et al.* 1999; Bakiri *et al.* 2000; Nagata *et al.* 2001; Boulon *et al.* 2002; Klein & Assoian 2008; Witzel *et al.* 2010). Over-expression of cyclin D1 is thought to enhance cell cycle progression from G1 to S phase and increase cell proliferation. The cyclin/CDK kinase complexes also target substrates that play important roles in centrosome duplication, mitochondrial function, cell growth, cell adhesion and motility, and cytoskeletal modeling. Therefore, elucidation of the transcriptional regulation of *cyclin D1* is essential to understand its role in the tumorigenesis of NSCLC (Musgrove *et al.* 2011).

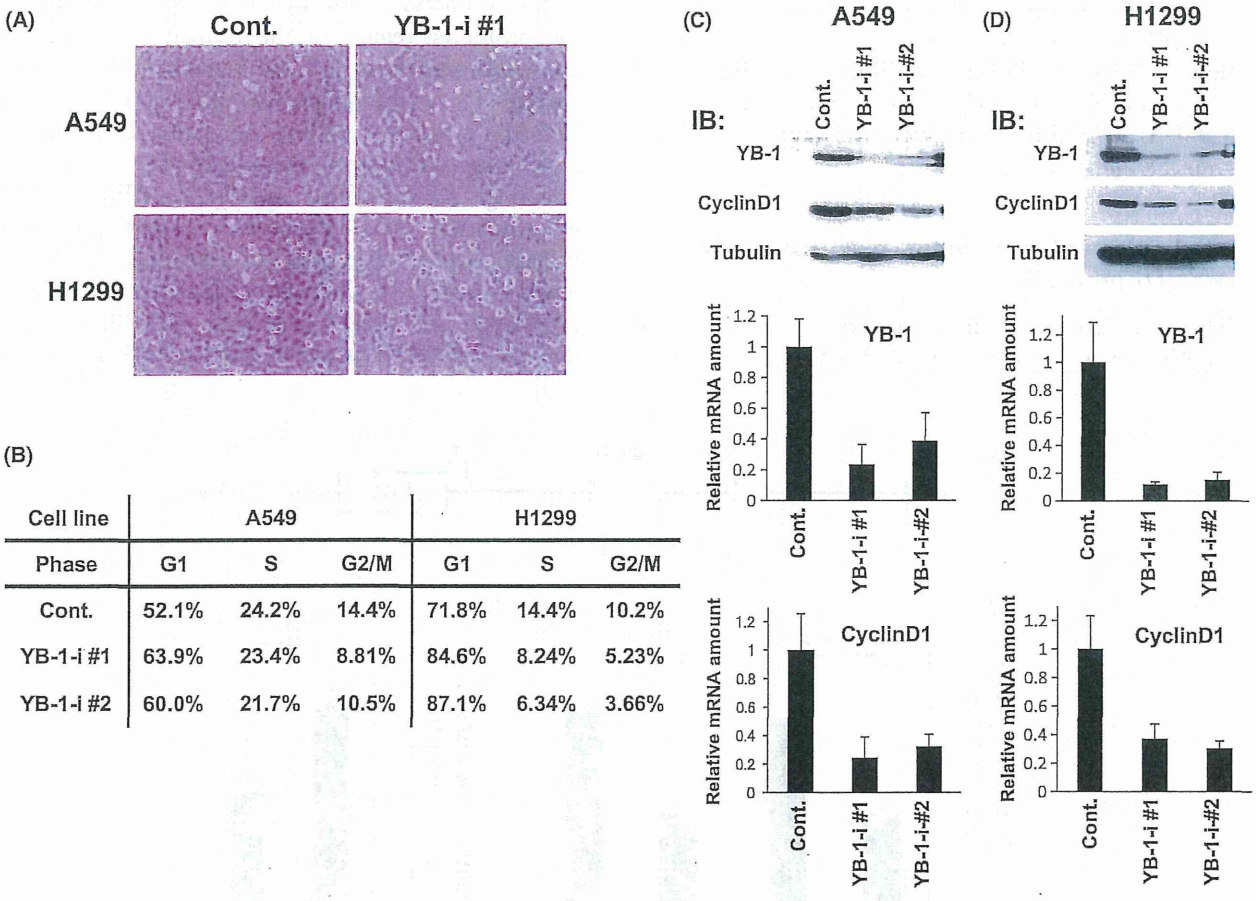
The Y-box-binding protein 1 (YB-1) is a member of the cold-shock domain protein superfamily that binds to an inverted CCAAT box, named the Y-box sequence, in the promoter regions of target genes. YB-1 is a multifunctional protein and regulates translation and transcription in the nucleus and cytoplasm. YB-1 has been reported to be a negative prognostic factor for several cancers, including breast, ovarian, and lung cancers and synovial sarcoma (Kohno *et al.* 2003). YB-1 has also been shown to up-regulate the transcription of cell-cycle-related molecules, including *cyclin A*, *cyclin B* (Jurchott *et al.* 2003), and *CDC6* (Basaki *et al.* 2010). However, we recently showed that YB-1 binds and represses the CDK inhibitor $p16^{ink4}$ gene (Kotake *et al.* 2013). This implies that YB-1 participates in cell cycle progression both via positive and negative regulatory pathways, that is, functioning as both an accelerator and a brake.

In lung cancer, increased expressions of not only YB-1 but also cyclin D1 were found independently, but the correlated expression and functional relationship between YB-1 and cyclin D1 have never been addressed in lung cancer (Eliseeva *et al.* 2011; Lasham *et al.* 2013). In the present study, we investigated whether YB-1 controls cyclin D1 expression in human lung cancers, with particular focus on our identification of several Y-boxes in the *cyclin D1* promoter. Moreover, we also investigated the correlation of CDC6, another target of YB-1, with expression of cyclin D1 and YB-1 in clinical samples of NSCLC.

Results

Effects of YB-1 knockdown in lung cancer cell lines

Based on the reports suggesting that YB-1 promotes cell cycle progression, we first evaluated whether the cell cycle of human lung cancer cell lines was positively regulated by YB-1. We investigated the effects of YB-1 knockdown on cellular proliferation of two lung cancer cell lines (A549 and H1299) by transfecting cells with YB-1 siRNA (YB-1-i #1) for 48 h. The proliferation of A549 and H1299 cells was suppressed to 41.9% and 45.7%, respectively, by YB-1 depletion (Fig. 1A), suggesting that YB-1 participates in the cell proliferation of these lung cancer cells. We next examined effects of YB-1 depletion on the cell cycle. A549 and H1299 cells were transfected with YB-1 siRNA (YB-1-i #1 or



(B)

Cell line	A549			H1299		
Phase	G1	S	G2/M	G1	S	G2/M
Cont.	52.1%	24.2%	14.4%	71.8%	14.4%	10.2%
YB-1-i #1	63.9%	23.4%	8.81%	84.6%	8.24%	5.23%
YB-1-i #2	60.0%	21.7%	10.5%	87.1%	6.34%	3.66%

Figure 1 Effect of YB-1 knockdown on cell proliferation, and expression of cyclin D1 protein and mRNA in non-small-cell lung cancer cells. (A) The cell proliferations of A549 and H1299 cells were observed by phase-contrast microscopy 48 h after treatment with YB-1 siRNA (right panels) or control siRNA (left panels). (B) A549 cells and H1299 cells were analyzed by flow cytometry 72 h post-transfection with two kinds of YB-1 siRNAs or control siRNA. The proportions of cell fractions in the different cell cycle phases are shown. (C) A549 cells and (D) H1299 cells were incubated with either YB-1 or control siRNA for 72 h, and lysates and total RNA were prepared. (Top panels) Western blot analysis for YB-1 and cyclin D1 proteins. α-tubulin served as a loading control. (Middle and bottom panels) Middle panel shows YB-1 relative mRNA amount levels, and bottom panel shows cyclin D1 relative mRNA amount levels as measured by qRT-PCR. Columns represent the mean of three independent experiments, and bars indicate SD.

YB-1-i #2) for 72 h and analyzed using flow cytometry. There was an increase in the proportion of cells in G1 phase (from 52.1% to 63.9% in A549 cells and from 71.8% to 87.1% in H1299 cells) and a decrease in the cells in S and G2/M phases, compared to control-siRNA-treated cells (Fig. 1B, Fig. S2 in Supporting Information). This result suggested that YB-1 might participate in the G1/S transition in lung cancer cells.

Effects of YB-1 knockdown on expression of cyclin D1 in NSCLC

Because cyclin D1 is involved in G1 progression, and as cyclin D1 contains several Y-box sequences in its promoter (see following section), we investigated whether expression of cyclin D1 was affected by YB-1 depletion in various lung cancer cell lines. Depletion of YB-1 by both YB-1-i #1 and #2 siRNA suppressed the protein levels of cyclin D1 in A549 and H1299 cells (Fig. 1C,D). Moreover, expression of cyclin D1 mRNA was also decreased in the two lung cancer cell lines treated with YB-1 siRNA compared with cells treated with control siRNA (Fig. 1C,D). The main histological findings in human lung cancers are adenocarcinoma and squamous cell carcinoma, so other types of human lung cancer cell lines (ABC1: adenocarcinoma cell line, EBC1: squamous cell carcinoma cell line) were also investigated.

As with A549 and H1299 cells, cyclin D1 protein and mRNA expression were also decreased by depletion of YB-1 in ABC1 and EBC1 cells (Fig. S3 in Supporting Information). These results suggested that YB-1 is a positive transcriptional regulator of the *cyclin D1* gene in NSCLC.

YB-1 enhanced transcriptional activity of the *cyclin D1* promoter

YB-1 was previously found to bind to the inverted CAAT box sequences, named the Y-box sequences, in the promoter region of target genes. We searched the promoter region of the human *cyclin D1* gene and confirmed several Y-box sequences, as shown in Fig. 3A. To elucidate whether YB-1 positively regulates the promoter activity of *cyclin D1*, luciferase assays were conducted using a *cyclin D1* reporter plasmid (Fig. 2A). Luciferase activity in cells cotransfected with the YB-1 expression vector was almost twofold higher than that in cells transfected with empty vector in both human embryonic kidney cells (HEK293) and A549 cells (Fig. 2B). Furthermore, depletion of YB-1 in A549 cells suppressed the *cyclin*

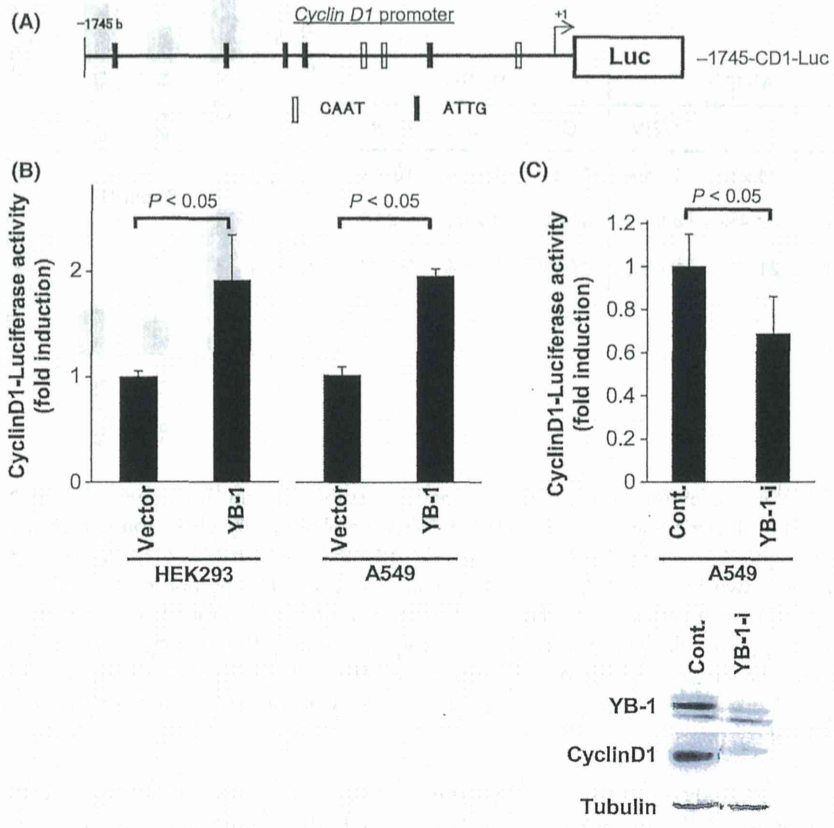


Figure 2 YB-1 enhanced the transcriptional activity of cyclin D1. (A) Schematic representation of the *cyclin D1* 5′ promoter construct in the pSPLUC vector. Potential YB-1-binding sites (Y-boxes), ATTG (black box) or CAAT (open box), are shown. (B) Luciferase assays using the *cyclin D1* reporter in HEK293 and A549 cells. Cells were transfected in Opti-MEM medium with the cyclin D1 promoter luciferase reporter plasmid, CMV β-gal plasmid, and either pcDNA3.1-YB-1 plasmid or pcDNA3 empty vector. After 48 h of incubation, lysates were prepared and luciferase activities were evaluated. Luciferase activity was normalized to β-gal activity, which was assayed in parallel. Luciferase activities of the pcDNA3-YB-1 transfected sample were presented as the relative ratio to pcDNA3 empty vector-transfected sample. Data represent the mean from three experiments. (C) Luciferase assays using the *cyclin D1* reporter in A549 cells. A549 cells were transfected in Opti-MEM medium with the cyclin D1 promoter luciferase reporter plasmid, CMVβ-gal plasmid, and either YB-1 or control siRNA. (Top panel) After 48 h incubation, lysates were prepared. Luciferase activities were normalized to parallel assays for β-gal activities. Luciferase activities of the YB-1 siRNA-transfected sample were presented as the relative ratio to control siRNA-transfected sample. Data represent the mean from three experiments. (Bottom panel) Lysates from the luciferase assay were analyzed by Western blot analysis.

D1 promoter activity (Fig. 2C). Protein expression levels of YB-1 and cyclin D1 were also verified by immunoblotting (Fig. 2C). These results suggested that YB-1 positively regulated *cyclin D1* promoter activity, indicating that YB-1 increases *cyclin D1* mRNA levels, promoting the increase of cyclin D1 protein and subsequently driving G1/S cell cycle transition.

YB-1 directly binds the promoter region of *cyclin D1* gene

We next investigated the mechanism underlying the activation of *cyclin D1* transcription. In previous studies, YB-1 was shown to directly bind the Y-box sequence of several promoters, including *EGFR Her2* (Wu *et al.* 2006), *CDC6* (Basaki *et al.* 2010), and *E2F* (Lasham *et al.* 2012). We searched the first 6 kb of the *cyclin D1* promoter and identified 19 putative YB-1-responsive elements. We next performed chromatin immunoprecipitation (ChIP) assays to determine whether YB-1 directly binds the *cyclin D1* promoter in NSCLC cells. A schematic representation of the potential YB-1 binding sites and the locations of primers used for the ChIP assay in the *cyclin D1* promoter is shown in Fig. 3A. First, we designed five primer sets, 'a' (−4762 to −4501 bp), 'b' (−2341 bp to −2048 bp), 'c' (−1539 bp to −1273 bp), 'd' (−1132 bp to −890 bp) and 'e' (−542 bp to −278 bp), and conducted the ChIP assay. As shown in Fig. 3B, endogenous YB-1 in the A549 lung cancer cell line bound the promoter of *cyclin D1* within the primer sets 'b' and 'c', with particularly strong binding to the 'c' region. Semi-quantitative evaluation is also shown in Fig. 3C. This result suggested that the region around the primer set 'c' was the best candidate for the YB-1 binding site. To clarify the binding region of endogenous YB-1 to *cyclin D1* promoter, we designed additional primer sets, 'f' (−1478 bp to −1114 bp) and 'g' (−1294 bp to −890 bp; Fig. 3A). Results confirmed that endogenous YB-1 also bound to the *cyclin D1* promoter within the regions for the primer sets 'f' and 'g', with stronger binding to the 'g' region than the 'f' region (Fig. 3B). To further confirm the binding of endogenous YB-1 to the *cyclin D1* promoter in NSCLC cells, we depleted endogenous YB-1 by siRNA and carried out the ChIP assay. We confirmed that YB-1 siRNA effectively depleted YB-1 protein and thereby cyclin D1 protein level was down-regulated (Fig. 3D). The binding abilities of endogenous YB-1 to the *cyclin D1* promoter in primer regions 'c' and 'f' were canceled by YB-1 knockdown, but not in 'b' and 'g' (Fig. 3E,F). As depletion of YB-1 by siRNA specific for YB-1 could not eliminate the band from the 'g' region, we speculate that other transcription factors, including another Y-box-binding protein family member, that bind to the 'b' and 'g' region might cross-react with the YB-1 polyclonal antibody used in the ChIP assay. These results were reproducible (data not shown). We conclude that 'c' and 'f' but not 'g' are the specific binding sites for YB-1 in the *cyclin D1* promoter. These data indicated that endogenous YB-1 binds the promoter of *cyclin D1* around −1539 to −1114 from the transcription start site. In summary, these results suggest that YB-1 binds to the *cyclin D1* promoter near the transcription start site and positively regulates transcription of *cyclin D1*, increasing both cyclin D1 mRNA and protein levels, and promoting the G1/S cell cycle transition and cell proliferation in NSCLC cells.

YB-1 mRNA levels positively correlated to cyclin D1 mRNA levels in human NSCLC tissues

Next, we evaluated whether mRNA expression of YB-1 positively correlated to that of cyclin D1 in human NSCLC tissues. Total RNAs were prepared from clinical frozen samples of 34 patients with NSCLC. Amounts of both YB-1 and cyclin D1 mRNA were measured using real-time RT-qPCR. As shown in Fig. 4A, expression of cyclin D1 mRNA positively correlated with YB-1 ($r = 0.3987$, $P = 0.0177$; Fig. 4A). This suggested that YB-1 participates in transcriptional control of *cyclin D1* mRNA expression in NSCLC.

Correlation of YB-1 protein expression with cyclin D1 and CDC6 in NSCLC clinical samples

Next we evaluated whether expression of YB-1 positively correlated with cyclin D1 at both the mRNA level and protein level. A recent study reported that the protein level of YB-1 correlated to CDC6 in lung and breast cancer cells (Basaki *et al.* 2010). Therefore, we determined whether the protein expression of YB-1 was associated with that of both cyclin D1 and CDC6 in 326 NSCLC tissue samples. Representative immunohistochemical staining of tissue microarrays (TMAs) with antibodies against YB-1, cyclin D1, and CDC6 is shown in (Fig. 4B). Expression of YB-1 was detected in 300 (92%) of 326 patients with NSCLC. The percentage of positively stained YB-1 and cyclin D1 cells in both

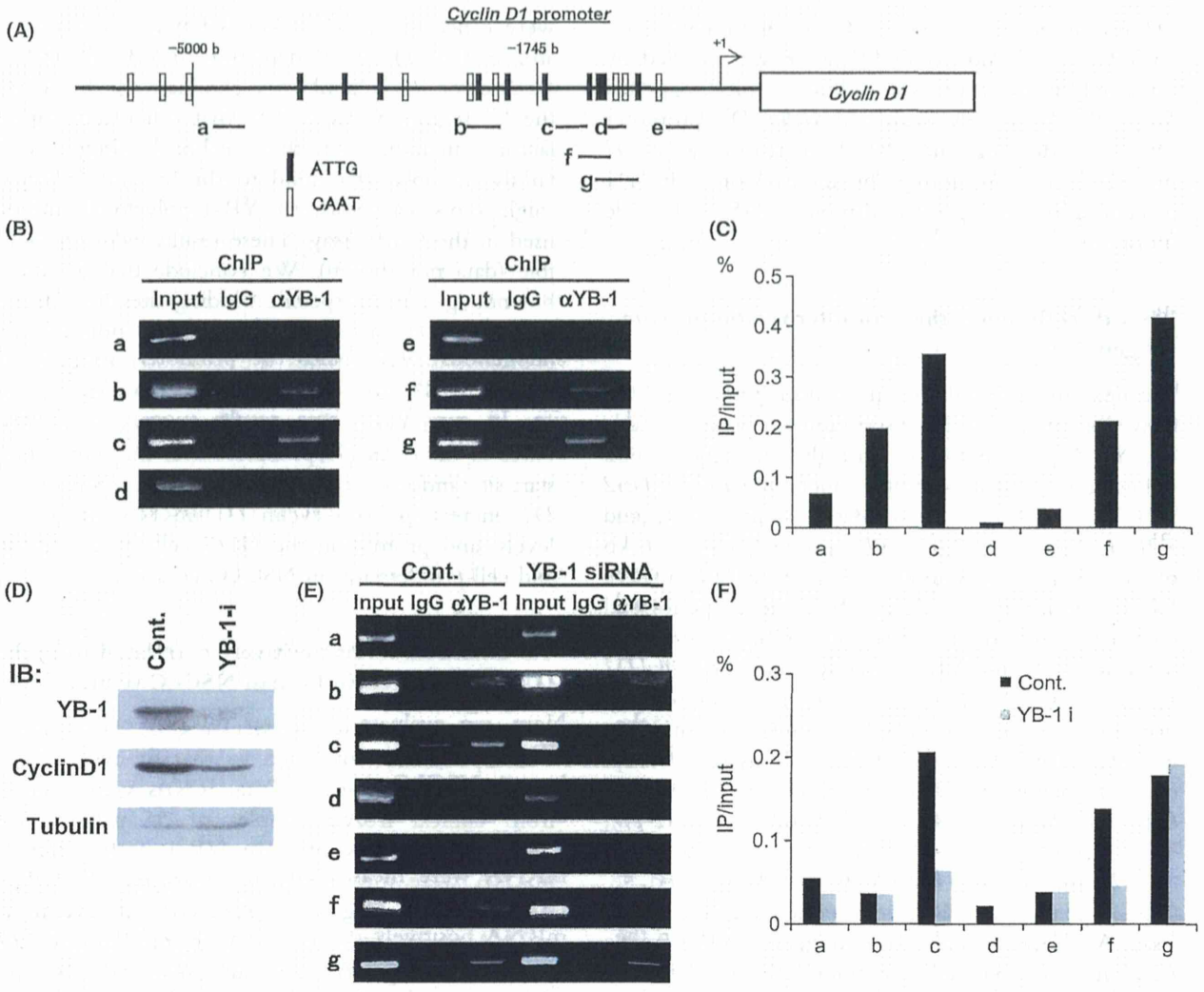


Figure 3 YB-1 directly binds the promoter region of cyclin D1. (A) Schematic representation of the potential YB-1 binding sites and the location of the primers used for chromatin immunoprecipitation (ChIP) in the promoter region of cyclin D1. Black bars (a–g) show regions for PCR primers. Black and open boxes indicate Y-boxes (CAAT box and ATTG box, respectively). (B) ChIP assays in A549 cells to confirm endogenous YB-1 binding to the cyclin D1 promoter were performed with an anti-YB-1 antibody (αYB-1) or normal rabbit IgG. DNA immunoprecipitated with either normal rabbit IgG (lanes 2) or anti-YB-1 antibody (lanes 3), and input chromatin (lanes 1) was amplified by PCR with the specific primers (a–g) for the cyclin D1 promoter. (C) Relative amounts of the binding to the cyclin D1 promoter for each primer set to input were quantified. (D–F) A549 cells were treated with YB-1 or control siRNA in Opti-MEM medium for 72 h, and cells were harvested and evaluated by ChIP assay. The results of the immunoblotting are shown in (D). Results of the ChIP assay are indicated in (E), and relative amounts of the binding to the cyclin D1 promoter are quantified in (F).

normal and tumor tissues is shown in Fig. 4C. Among normal lung tissues, few normal lung tissue cases showed expression of YB-1 and/or cyclin D1. In contrast, YB-1 and cyclin D1 were both expressed in most human NSCLC tissue cases. Table 1 shows the results of Fisher's exact test for association between YB-1 and cyclin D1 or CDC6 in NSCLC tissues. There was a significant correlation between expression of YB-1 and cyclin D1 ($P = 0.0033$), and between expression of YB-1 and CDC6 ($P = 0.0045$). These results suggested that YB-1 and cyclin D1 were highly expressed in NSCLC tissues and indicate that YB-1 correlates positively to cyclin D1 in NSCLC tissues. Next, we investigated the correlation of YB-1 to cyclin D1 and CDC6 in each patient. YB-1 expression positively correlated to

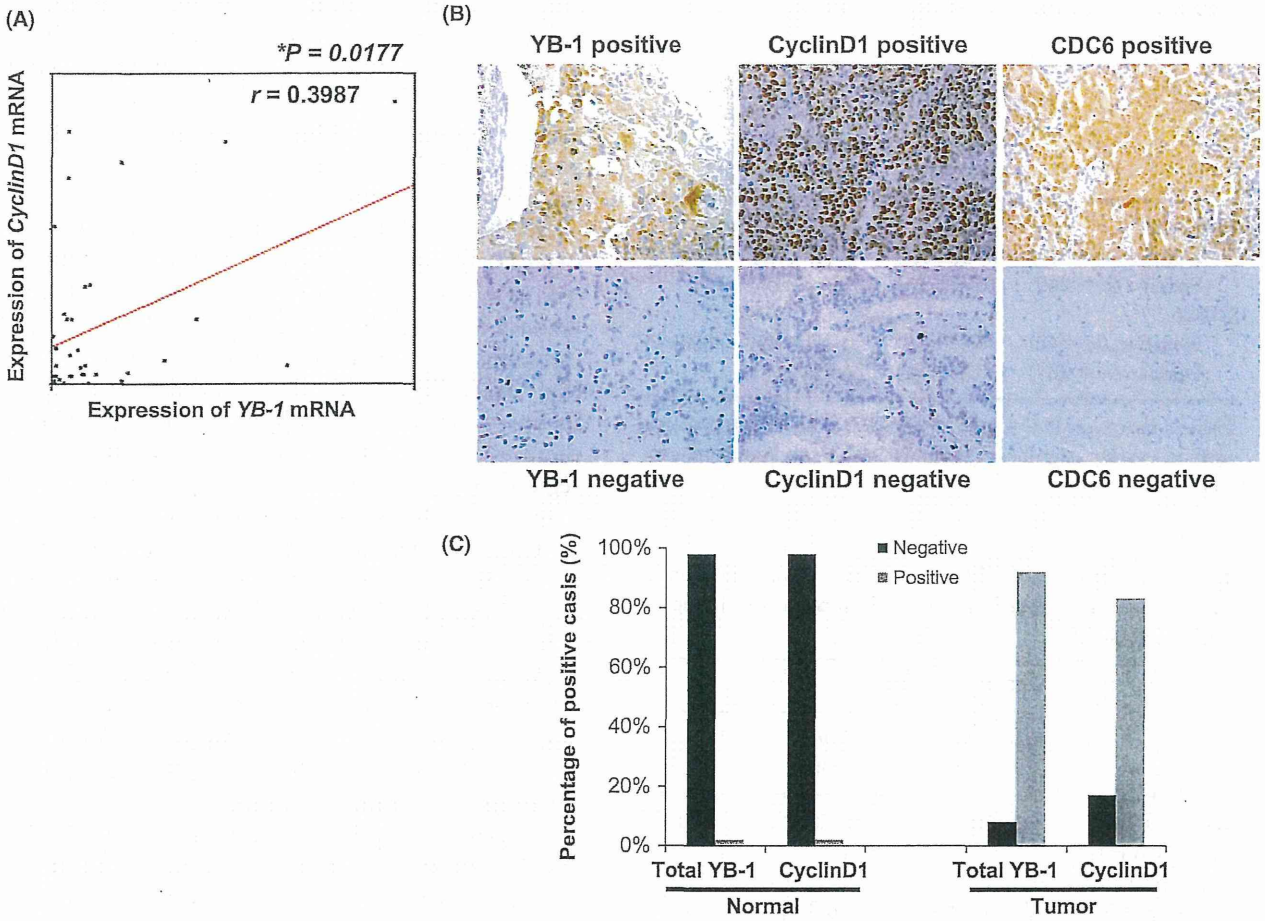


Figure 4 YB-1 and cyclin D1 mRNA and protein expression in non-small-cell lung cancer (NSCLC) clinical samples. (A) The expression of YB-1 and cyclin D1 mRNA was analyzed using RT-qPCR in 34 patients with NSCLC. To determine whether there is a positive correlation between total mRNA expression of YB-1 and cyclin D1, total mRNAs were extracted from NSCLC tissues, and total YB-1 and cyclin D1 mRNA levels were measured as described in Materials and methods. Positive correlation was detected between YB-1 and cyclin D1 mRNA levels in NSCLC samples. Statistical analysis was evaluated by bivariate correlation analysis. '*r*' indicates the correlation coefficient. (B,C) Histological findings, and expression of YB-1, cyclin D1 and CDC6 in 326 NSCLC samples. Representative samples of positive and negative findings are shown in (B) (magnification ×400). These positive expression samples for YB-1, cyclin D1, and CDC6 were classified as 'score +2'. The mean percentage of the cell population with positive expression among five fields of YB-1 and cyclin D1 is shown in (C). The left graph reflects YB-1 and cyclin D1 protein expression in normal cells, and the right graph indicates levels in cancer cells in NSCLC samples.

both cyclin D1 and CDC6 expression ($P = 0.0014$; Table 2). Therefore, these results suggested that YB-1 positively regulates both cyclin D1 and CDC6 at the same time, effectively leading to enhanced G1 to S transition.

Discussion

Cyclin D1 is involved in the G1/S cell cycle transition, and its deregulated over-expression is often found not only in breast cancer but also in other various human cancers, including lung cancers (Kim & Diehl 2009). Over-expression of cyclin D1 leads to excess activation of cyclin D1-CDK4/6 and enhanced phosphorylation of Rb protein, resulting in the abrogation of the Rb tumor suppressor pathway. Cyclin D1 over-expression is associated with carcinogenesis, cancerous growth, and malignant phenotypes (Kim & Diehl 2009). Transcription of cyclin D1 is promoted by transcriptional factors such as TCF/LEF, Jun, NFκB, STAT3/5, EtsB, and more (Klein & Assoian 2008). Here, we showed that cyclin D1

Table 1 Correlation of YB-1 expression to cyclin D1 or CDC6 expression

	YB-1 (n = 326)		
	Negative (n = 26)	Positive (n = 300)	P
Cyclin D1			
Negative (n = 57)	10 (38.5%)	47 (15.7%)	0.0033**
Positive (n = 269)	16 (61.5%)	253 (84.3%)	
CDC6			
Negative (n = 42)	8 (30.8%)	34 (11.3%)	0.0045**
Positive (n = 284)	18 (69.2%)	266 (88.7%)	

Fisher's exact test (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$) was used to evaluate significant difference. Data are presented as n (%).

Table 2 Correlation of YB-1 expression to cyclin D1 and CDC6 expression in each patient with non-small-cell lung cancer (NSCLC)

	YB-1 (n = 326)		
	Negative (n = 26)	Positive (n = 300)	P
Cyclin D1 (+) + CDC6 (+) (n = 247)	13 (50.0%)	234 (78.0%)	0.0014**
Others (n = 79)	13 (50.0%)	66 (22.0%)	

Fisher's exact test (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$) was used to evaluate significant difference. Data are presented as n (%). Cyclin D1 (+) and CDC6 (+) indicate positive expression of both cyclin D1 and CDC6. 'Others' refer to cyclin D1 (+) and CDC6 (−), cyclin D1 (−) and CDC6 (+), and cyclin D1 (−) and CDC6 (−).

expression is positively controlled by YB-1 and is one of the transcriptional targets for YB-1 in human lung cancers. Our results suggest that one of the mechanisms of aberrant expression of cyclin D1 is due to over-expression of YB-1 in NSCLC.

The tumor suppressor p53 is frequently inactivated in multiple human cancers, including lung cancers (Sun 2006). YB-1 has been reported to interact directly with p53, and YB-1 reduced the p53-driven transcriptional activation of apoptosis-associated genes *APAF1* (apoptotic peptidase activating factor 1), *NOXA* (NADPH oxidase activator), and *BAX* (Bcl2-associated X protein) (Okamoto *et al.* 2000; Homer *et al.* 2005). In this study, YB-1 depletion promoted G1 arrest and suppressed cyclin D1 expression in not only A549 cells, which express wild-type p53, but also H1299, which has a homozygous partial deletion of the *p53* gene. Moreover, cyclin D1 expression was also suppressed in ABC1 and EBC1 cells, which both express mutant p53. These results suggest that YB-1 promotes cyclin D1 expression regardless of the p53 status in lung cancers.

We found that endogenous YB-1 bound to the *cyclin D1* promoter in the region around −1539 bp to −1114 bp, as depletion of YB-1 abolished the binding abilities of YB-1 to regions in the *cyclin D1* promoter corresponding to primer sets 'c' and 'f', but not others (Fig. 3E,F). We identified a cluster of Y-box sequences in the 'f' region (−1478 bp to −1114 bp), although there is no Y-box sequence within primer set 'c' region (−1539 bp to −1273 bp). Long chromatin fragments with the Y-box region that is outside of the primer set 'c' region may be in the chromatin fraction and therefore it was able to be detected by the primer 'c' set in the ChIP assay. Dolfini & Mantovani (2012) suggested Y-box-independent binding of YB-1 to the *cyclin D1* promoter; therefore, another possibility is that YB-1 may bind to the *cyclin D1* promoter around −1539 to −1273 in a Y-box-independent manner. Further studies to fully elucidate YB-1 interactions on the *cyclin D1* promoter are required.

Several transcriptional factors, including TCF/LEF, CREB, NF-κB, AP-1, and SP1, have been reported to transactivate the *cyclin D1* promoter. YB-1 also forms a transcriptional activation complex with RARα, KLF4, and Sp1 on the *Klf4* promoter (Shi *et al.* 2012). Moreover, YB-1 binds p300 and forms a complex on the *MDR1* promoter to activate it (Sengupta *et al.* 2011). Therefore, YB-1 can bind co-activators and/or other transcription factors, and may thus collaboratively regulate transcription of YB-1 target genes including *cyclin D1* in a Y-box-dependent manner. It is also like that other transcription factors recruit YB-1 to their respective binding sequences and thus YB-1 may regulate the transcription of other genes in a Y-box-independent manner.

Our results indicated that cyclin D1 expression was positively correlated with YB-1 expression in clinical samples of NSCLC. We also investigated the correlation of CDC6, another target of YB-1, with expression of cyclin D1 and YB-1, and found that NSCLC samples expressing both cyclin D1 and CDC6 are significantly associated with over-expression of YB-1. CDC6 binds to the ORC–origin complex to form a pre-replication complex as an essential licensing factor for DNA replication initiation (Sacco *et al.* 2012). Previous reports have suggested that

over-expression of CDC6 enhances re-replication, leading to genomic instability, and that CDC6 is involved not only in DNA replication but also in transcriptional regulation (Petrakis *et al.* 2012). Over-expression of CDC6 participates in the transcriptional repression of the tumor suppressor *INK4/ARF* locus via histone deacetylase recruitment to and heterochromatization of the *INK4/ARF* locus (Gonzalez *et al.* 2006). The *INK4/ARF* locus encodes the CDK inhibitor $p15^{ink4b}$ and $p16^{ink4a}$, and the Mdm2 inhibitor ARF, which contribute to the tumor suppressive Rb and p53 pathways, respectively (Gil & Peters 2006). Moreover, we recently reported that YB-1 binds the $p16^{ink4a}$ gene and suppresses its transcription. Therefore, YB-1 promotes aberrant activation of cyclin D1-CDK4/6 to abrogate the Rb pathway via both over-expression of cyclin D1 and suppression of Ink4-type CDK inhibitors. Altogether, aberrant expression of both cyclin D1 and CDC6 mediated by YB-1 over-expression may effectively promote lung carcinogenesis.

We confirmed that YB-1 promoted transcriptional activation of *cyclin D1* promoter via a luciferase reporter system using human lung cancer cells as well as HEK293 cells, a human embryonic kidney cell line. Therefore, we speculate that YB-1 is involved in regulating the expression of cyclin D1 in cells expressing YB-1, and that YB-1 does not specifically function only in human lung cancer. However, whether the same mechanism of YB-1 regulation of cyclin D1 expression is involved in the development of other human cancers should be determined. Over-expression of YB-1 has been reported in various human cancers (Lasham *et al.* 2013). Transcription of YB-1 is promoted by Twist (Shiota *et al.* 2008) and GATA-1/2 (Yokoyama *et al.* 2003), and over-expression of both is found in several human cancers. Moreover, mutual activation of the RAS/RAF/MAPK cascade promotes YB-1 expression and thereby expression of EGFR and ERK2, and phosphorylation-mediated activation of MEK and ERK are promoted as an autostimulating cascade (Imada *et al.* 2013). Because aberrant activations of B-RAF, including the V600E mutation, are frequently found in lung cancer (Marchetti *et al.* 2011; Oxnard *et al.* 2013), activated B-RAF-mediated MAPK activation may participate in over-expression of YB-1 in lung cancers. Chemical inhibitors against B-RAF may be effective against YB-1-associated lung cancer to suppress YB-1 expression and thus expressions of both cyclin D1 and CDC6 may be down-regulated. This speculation is consistent with our result that depletion of YB-1 by siRNA arrested lung cancer cell lines in G1 phase regardless of the p53 status.

Experimental procedures

Cell culture

The human lung cancer cell lines A549, H1299, ABC1 and EBC1, and human embryonic kidney HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and maintained at 37 °C in an atmosphere containing 5% CO_2. EBC1 was obtained from the RIKEN Cell Bank (Tsukuba, Japan).

Antibodies

The antibodies used in this study were as follows: anti-YB-1 antibody (Abcam, Cambridge, UK), anti-cyclin D1 antibody (Santa Cruz, CA, USA), anti-CDC6 antibody (ProteinTech Group Inc., Chicago, IL, USA), and anti-α-tubulin antibody DM1A (Sigma).

Plasmids and recombinant proteins

A reporter construct containing −1745 bp of the human *cyclin D1* promoter linked to a luciferase reporter gene (-1745-CD1-Luc) was kindly provided by Dr. Suzuki (Department of Oncology, Institute of Medical Science, The University of Tokyo, Tokyo, Japan). A plasmid containing the full-length cDNA fragment of human YB-1 was generated by reverse transcription PCR using total RNA from A549 cells. Reverse transcription was carried out with random hexanucleotide primers and SuperScript Reverse Transcriptase II (Invitrogen, California, USA). To construct the YB-1 expression plasmid, the BamH1 fragment of the YB-1 cDNA was cloned into the pcDNA3.1 plasmid. All plasmids were constructed using standard recombinant DNA techniques. Primer sequences of the full-length cDNA fragment of human YB-1 were 5′-ACTGG ATCCATGAGCAGCCGAGGCCGAGACC-3′ and 5′-ACT GGATCCTTACTCAGCCCCGCCCTGCTC-3′.

RNA interference

Cells were transfected with human *YB-1* siRNA or control siRNA oligonucleotides using Lipofectamine™ RNAiMAX (Invitrogen), according to the manufacturer's protocol. The nucleotide sequence of the *YB-1*-i-#1 siRNA was 5′-UGAC ACCAAGGAAGAUGUA-3′, and *YB-1*-i-#2 siRNA was 5′-G UGAGAGUGGGGAAAAGAA-3′ with a 3′ dTdT overhang.

Immunoblot analysis

Cells were lysed in lysis buffer (0.3% Triton X-100, 300 mM NaCl, 50 mM Tris-HCl, pH 7.5). Cell lysates were denatured

by treatment with SDS sample buffer at 95 °C for 8 min. Cell lysates were separated by SDS–PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), followed by immunoblotting. The proteins were visualized using an enhanced chemiluminescence system (Perkin Elmer, Waltham, MA, USA). The antibodies we used included anti-cyclinD1 (DCS-6; Santa Cruz Biotechnology), anti-human YB-1 (ab12148; Abcam), and anti-α-tubulin (Sigma). Primary antibodies for anti-human YB-1 and anti-human cyclinD1 were used at a final dilution of 1 : 1000.

Quantitative reverse transcriptional polymerase chain reaction analysis

Total RNA was isolated from cultured cells and human clinical frozen samples using RNase Plus mini kit (Qiagen) for culture cells, and an Isogen kit (Wako, Osaka, Japan) for human clinical samples according to the manufacturers' instructions. Reverse transcription was carried out with random hexanucleotide primers and SuperScript Reverse Transcriptase II (Invitrogen). The resulting cDNA was subjected to real-time polymerase chain reaction using the Rotor-Gene 3000 System (Corbett Research, Mortlake, Australia) and a QuantiTect SYBR Green PCR kit® (Qiagen, Valencia, CA, USA) or SYBR® Green Real-time PCR Master Mix (TOYOBO CO., Osaka, Japan). Primer sequences were 5′-AAGTGATGGAGGGTGCTGAC-3′ and 5′-TTCTTCATTGCCGTCCTCTC-3′ for YB-1, and 5′-GCTCCTGTGCTGCGAAGT-3′ and 5′-TGTTCCTCGCAGACCTCCAG-3′ for cyclin D1. Transcripts were normalized to 18S rRNA mRNA.

Luciferase reporter assay

Cells (1.0×10^5 per well) cultured in six-well plates were transfected with 0.5 μg of the luciferase reporter plasmid, 100 ng of the CMV-β-gal plasmid, and 1 μg (or the indicated amount) of human YB-1 expression vector or 1 μg empty vector, using Fugene6® reagent (Promega) in Opti-MEM (Invitrogen). Cells were lysed 48 h after transfection, and assayed for luciferase and β-galactosidase activities, with the former being normalized by the latter.

ChIP assay

A549 cells (4.4×10^6) were treated with 1% formaldehyde, and cross-linking was stopped by the addition of 0.125 M glycine. The cells were lysed with cell lysis buffer on ice (10 mM Hepes/pH 7.9, 0.5% NP-40, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and protease inhibitor cocktail). After centrifugation, the cell pellets were lysed by sonication on ice with nuclear lysis buffer (20 mM Hepes/pH 7.9, 25% glycerol, 0.5% NP-40, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and protease inhibitor cocktail). After centrifugation, the lysates were diluted with equal volume of dilution buffer (1% Triton X-100, 2 mM EDTA, 50 mM NaCl, 20 mM Tris-HCl/pH 7.9, and protease inhibitor cocktail). Immunoprecipitation was carried out with an antibody specific to YB-1 antibody (BML) and normal rabbit IgG as a control. After immunoprecipitation, 20 μL salmon sperm DNA/protein G agarose (Millipore) was added, followed by 1-h incubation. The eluates were incubated at 65 °C to reverse the formaldehyde cross-linking. DNA fragments were purified using a PCR purification kit (Qiagen). PCR was carried out using Platinum Taq polymerase (Invitrogen) and the following pairs of primers: *cyclin D1* (a), 5′-CGGACAGTCGCCTTATTACG-3′ and 5′-CCTGCGTCCGCGTTTACC-3′; (b), 5′-AAAATCAAAATGCTTTCTCTGC-3′ and 5′-GGGCAGATCTCGACTAGGAAC-3′; (c), 5′-GGAGGAATTCACCCTGAAAG-3′ and 5′-ACAGGTTCTGTCTCTTTGGTG-3′; (d), 5′-GAATTATGCCGGCTCCTG-3′ and 5′-TTAACCGGGAGAAACACACC-3′; (e), 5′-AAAGAAGATGCAGTCGCTGAG-3′ and 5′-CAGGGAAGAGGGGTGCAG-3′; (f), 5′-CGAAGGGGAGAGGGCTTT-3′ and 5′-CAGGAGCCGGCATAATTC-3′; (g), 5′-CACCAAAGAGACAGAACCTGT-3′ and 5′-TTAACCGGGAGAAACACACC-3′. The PCR programme was set with an initial melting step at 94 °C for 2 min, then 34 cycles of (94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s). The PCR products were then analyzed on agarose gel by electrophoresis.

Cell cycle analysis

A549 and H1299 cells (8.0×10^5) were transfected with YB-1 siRNA or control siRNA for 72 h, and 2.0×10^6 cells were evaluated. Cells were harvested after trypsin treatment and fixed with 70% ethanol in phosphate-buffered saline (−). Cells were incubated with RNase at 37 °C for 20 min and treated with 20 μg/mL of propidium iodide. DNA content and BrdU incorporation were analyzed by flow cytometry.

Patient characteristics and tissue specimens

Specimens of lung cancers were obtained from 326 Japanese patients with primary NSCLC whose tumors had been completely surgically removed in the Department of Surgery of Hamamatsu University School of Medicine between 1988 and 2007. Among the 326 patients, 197 patients were diagnosed histologically as having adenocarcinoma, and 110 patients were diagnosed as having squamous cell carcinoma. The other patients were diagnosed as having large cell carcinoma. The age of the patients with NSCLC ranged from 26 to 86 years (median, 66 years). Of the total number of patients, 222 were men and 104 were women. There were no significant differences in each parameter (Table S1 in Supporting Information).

This study was carried out in accordance with the guidelines of the Declaration of Helsinki, and the study protocol for this project was approved by the Research Ethics Committee of Hamamatsu University School of Medicine (Approved No. 23-91). A written letter of consent was