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

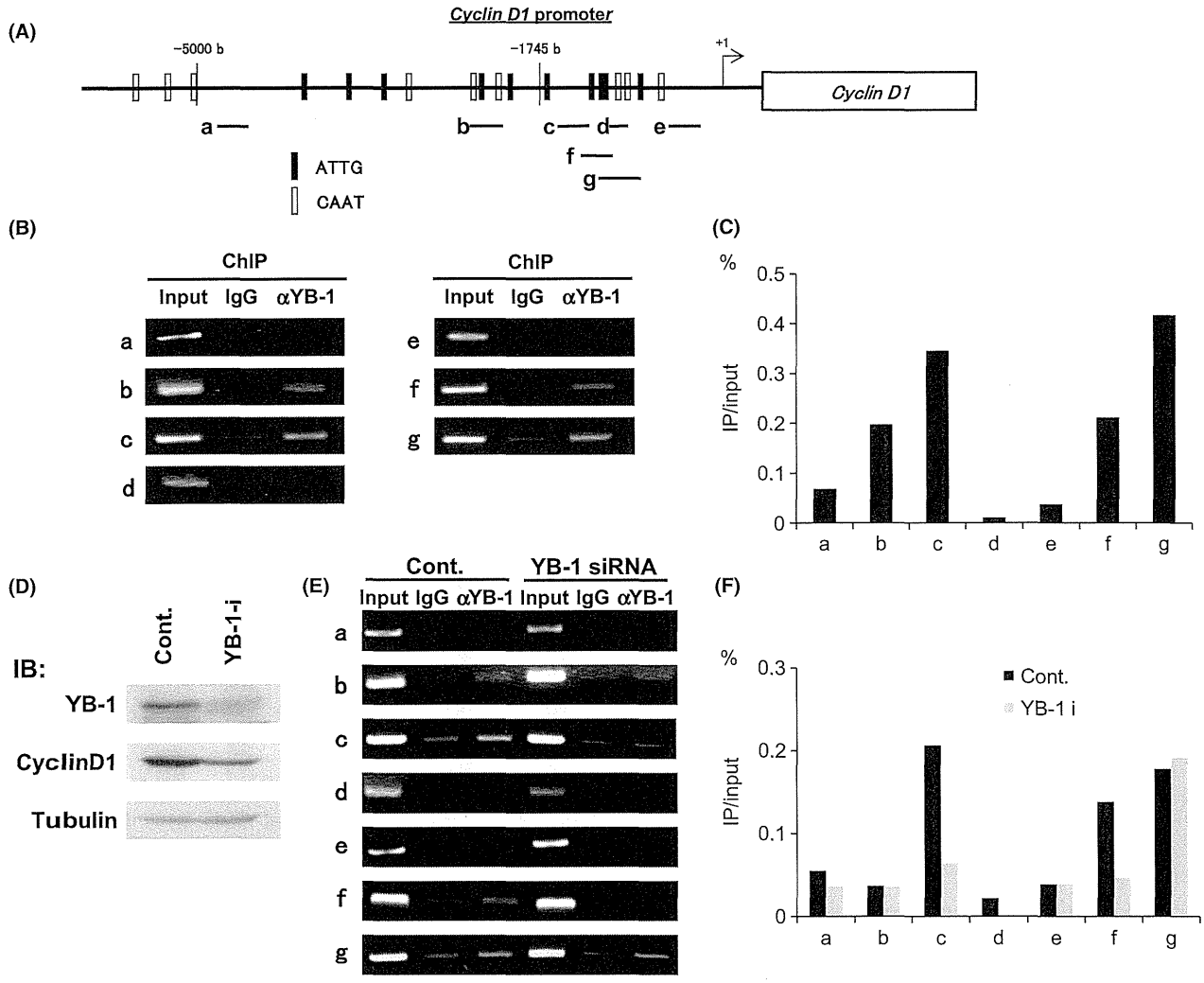

Figure 3 YB-1 directly binds the promoter region of cyclin D1. (A) Schematic representation of the potential YB-1 binding sites and the location of the primers used for chromatin immunoprecipitation (ChIP) in the promoter region of cyclin D1. Black bars (a–g) show regions for PCR primers. Black and open 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

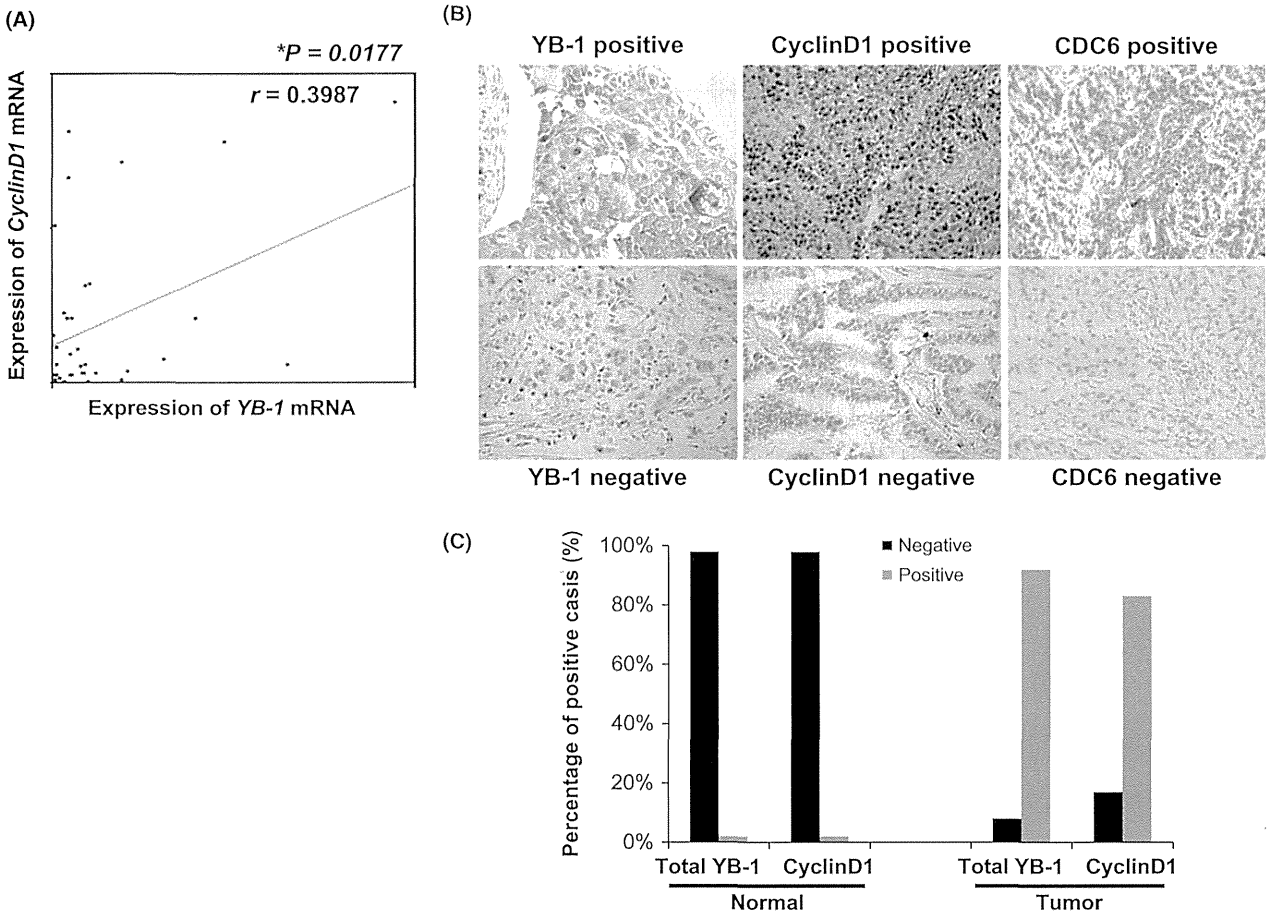

Figure 4 YB-1 and cyclin D1 mRNA and protein expression in non-small-cell lung cancer (NSCLC) clinical samples. (A) The expression of YB-1 and cyclin D1 mRNA was analyzed using RT-qPCR in 34 patients with NSCLC. To determine whether there is a positive correlation between total mRNA expression of YB-1 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 hetero-chromatization 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

processed after obtaining the patient informed consent to participate in this study.

Immunostaining

For immunohistochemical analysis, TMA blocks were prepared and then deparaffinized, rehydrated, and boiled for 30 min in Tris-EDTA buffer (pH 9.0) for CDC6 or 10 mM sodium citrate buffer (pH 6.0) for cyclin D1 and YB-1 as antigen retrieval (Igarashi *et al.* 1994; Sugimura 2008). Endogenous peroxidase activity was blocked by incubation in a hydrogen peroxide solution for 30 min. The sections were then incubated with a rabbit anti-YB-1 polyclonal antibody (Abcam, 1 : 7500), a rabbit anti-cyclin D1 monoclonal antibody (Nichirei, Tokyo, Japan, 1 : 25) and a rabbit anti-CDC6 polyclonal antibody (ProteinTech Group Inc., 1 : 100). The antigen-antibody complex was visualized using Histofine Simple Stain Max Po (Multi; Nichirei, Tokyo, Japan) and 3, 3′-diaminobenzidine tetrahydrochloride. Counterstaining was carried out using hematoxylin. For the evaluation of YB-1, cyclin D1, and CDC6 expression, the number of stained cells was counted, and at least five high-power fields were chosen randomly for scoring of the percentage of cells with positive staining among 1000 cells examined per section.

Expression of YB-1, cyclin D1, and CDC6 was classified into three categories: score 0, no staining at all or expression in <10% of cancer cells; score 1+, expression >10% and <50%; score 2+, expression >50%. The expressions of YB-1, cyclin D1, and CDC6 were defined as follows: scores 1+ and 2+ were regarded positive, and score 0 was regarded negative (Fig. S1 in Supporting Information).

Statistical analysis

Data are presented as mean ± SD. Data were analyzed by Student's *t*-test or Fisher's exact test, where $P < 0.05$ was considered to be statistically significant. All data analysis was carried out using a statistical software package JMP® software (version 10, SAS Institute Inc., Cary, NC, USA).

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References

Bakiri, L., Lallemand, D., Bossy-Wetzel, E. & Yaniv, M. (2000) Cell cycle-dependent variations in c-Jun and JunB phosphorylation: a role in the control of cyclin D1 expression. *EMBO J.* **19**, 2056–2068.

Basaki, Y., Taguchi, K.-I., Izumi, H., Murakami, Y., Kubo, T., Hosoi, F., Watari, K., Nakano, K., Kawaguchi, H., Ohno, S., Kohno, K., Ono, M. & Kuwano, M. (2010) Y-box binding protein-1 (YB-1) promotes cell cycle progression through CDC6-dependent pathway in human cancer cells. *Eur. J. Cancer* **46**, 954–965.

Boulon, S., Dantonel, J.-C., Binet, V., Vié, A., Blanchard, J.-M., Hipskind, R.A. & Philips, A. (2002) Oct-1 potentiates CREB-driven cyclin D1 promoter activation via a phospho-CREB- and CREB binding protein-independent mechanism. *Mol. Cell. Biol.* **22**, 7769–7779.

Ding, L., Getz, G., Wheeler, D.A., *et al.* (2008) Somatic mutations affect key pathways in lung adenocarcinoma. *Nature* **455**, 1069–1075.

Dolfini, D. & Mantovani, R. (2012) YB-1 (YBX1) does not bind to Y/CCAAT boxes in vivo. *Oncogene* **19**, 521.

Eliseeva, I.A., Kim, E.R., Guryanov, S.G., Ovchinnikov, L.P. & Lyabin, D.N. (2011) Y-box-binding protein 1 (YB-1) and its functions. *Biochemistry Mosc.* **76**, 1402–1433.

Gil, J. & Peters, G. (2006) Regulation of the INK4b-ARF-INK4a tumour suppressor locus: all for one or one for all. *Nat. Rev. Mol. Cell Biol.* **7**, 667–677.

Gonzalez, S., Klatt, P., Delgado, S., Conde, E., Lopez-Rios, F., Sanchez-Cespedes, M., Mendez, J., Antequera, F. & Serrano, M. (2006) Oncogenic activity of Cdc6 through repression of the INK4/ARF locus. *Nature* **440**, 702–706.

Guttridge, D.C., Albanese, C., Reuther, J.Y., Pestell, R.G. & Baldwin, A.S. (1999) NF-κB controls cell growth and differentiation through transcriptional regulation of cyclin D1. *Mol. Cell. Biol.* **19**, 5785–5799.

Homer, C., Knight, D.A., Hananeia, L., Sheard, P., Risk, J., Lasham, A., Royds, J.A. & Braithwaite, A.W. (2005) Y-box factor YB1 controls p53 apoptotic function. *Oncogene* **24**, 8314–8325.

Igarashi, H., Sugimura, H., Maruyama, K., Kitayama, Y., Ohta, I., Suzuki, M., Tanaka, M., Dobashi, Y. & Kino, I. (1994) Alteration of immunoreactivity by hydrated autoclaving, microwave treatment, and simple heating of paraffin-embedded tissue sections. *APMIS* **102**, 295–307.

Imada, K., Shiota, M., Kohashi, K., Kuroiwa, K., Song, Y., Sugimoto, M., Naito, S. & Oda, Y. (2013) Mutual regulation between Raf/MEK/ERK signaling and Y-Box-binding protein-1 promotes prostate cancer progression. *Clin. Cancer Res.* **7**, 7.

Jemal, A., Bray, F., Center, M.M., Ferlay, J., Ward, E. & Forman, D. (2011) Global cancer statistics. *CA Cancer J. Clin.* **61**, 69–90.

Jurchott, K., Bergmann, S., Stein, U., Walther, W., Janz, M., Manni, I., Piaggio, G., Fietze, E., Dietel, M. & Royer, H.D. (2003) YB-1 as a cell cycle-regulated transcription factor facilitating cyclin A and cyclin B1 gene expression. *J. Biol. Chem.* **278**, 27988–27996.

Kanie, T., Onoyama, I., Matsumoto, A., Yamada, M., Nakatsumi, H., Tateishi, Y., Yamamura, S., Tsunematsu, R., Matsumoto, M. & Nakayama, K.I. (2012) Genetic reevaluation of the role of F-box proteins in cyclin D1 degradation. *Mol. Cell. Biol.* **32**, 590–605.

Kim, J.K. & Diehl, J.A. (2009) Nuclear cyclin D1: an oncogenic driver in human cancer. *J. Cell. Physiol.* **220**, 292–296.

Klein, E.A. & Assoian, R.K. (2008) Transcriptional regulation of the cyclin D1 gene at a glance. *J. Cell Sci.* **121**, 3853–3857.

Kohno, K., Izumi, H., Uchiumi, T., Ashizuka, M. & Kuwano, M. (2003) The pleiotropic functions of the Y-box-binding protein, YB-1. *BioEssays* **25**, 691–698.

Kotake, Y., Ozawa, Y., Harada, M., Kitagawa, K., Niida, H., Morita, Y., Tanaka, K., Suda, T. & Kitagawa, M. (2013) YB1 binds to and represses the p16 tumor suppressor gene. *Genes Cells* **18**, 999–1006.

Lasham, A., Print, C.G., Woolley, A.G., Dunn, S.E. & Braithwaite, A.W. (2013) YB-1: oncoprotein, prognostic marker and therapeutic target? *Biochem. J.* **449**, 11–23.

Lasham, A., Samuel, W., Cao, H., Patel, R., Mehta, R., Stern, J.L., Reid, G., Woolley, A.G., Miller, L.D., Black, M.A., Shelling, A.N., Print, C.G. & Braithwaite, A.W. (2012) YB-1, the E2F pathway, and regulation of tumor cell growth. *J. Natl Cancer Inst.* **104**, 133–146.

Lee, R.J., Albanese, C., Stenger, R.J., Watanabe, G., Inghirami, G., Haines, G.K., Webster, M., Muller, W.J., Brugge, J.S., Davis, R.J. & Pestell, R.G. (1999) pp60v-src induction of cyclin D1 requires collaborative interactions between the extracellular signal-regulated kinase, p38, and Jun kinase pathways: a role for cAMP response element-binding protein and activating transcription factor-2 In pp60v-src signaling in breast cancer cells. *J. Biol. Chem.* **274**, 7341–7350.

Marchetti, A., Felicioni, L., Malatesta, S., Grazia Sciarrotta, M., Guetti, L., Chella, A., Viola, P., Pullara, C., Mucilli, F. & Buttitta, F. (2011) Clinical features and outcome of patients with non-small-cell lung cancer harboring BRAF mutations. *J. Clin. Oncol.* **29**, 3574–3579.

Molina, J.R., Yang, P., Cassivi, S.D., Schild, S.E. & Adjei, A.A. (2008) Non-small cell lung cancer: epidemiology, risk factors, treatment, and survivorship. *Mayo Clin. Proc.* **83**, 584–594.

Musgrove, E.A., Caldon, C.E., Barraclough, J., Stone, A. & Sutherland, R.L. (2011) Cyclin D as a therapeutic target in cancer. *Nat. Rev. Cancer* **11**, 558–572.

Nagata, D., Suzuki, E., Nishimatsu, H., Satonaka, H., Goto, A., Omata, M. & Hirata, Y. (2001) Transcriptional activation of the cyclin D1 gene is mediated by multiple cis-elements, including SP1 sites and a cAMP-responsive element in vascular endothelial cells. *J. Biol. Chem.* **276**, 662–669.

Okamoto, T., Izumi, H., Imamura, T., Takano, H., Ise, T., Uchiumi, T., Kuwano, M. & Kohno, K. (2000) Direct interaction of p53 with the Y-box binding protein, YB-1: a mechanism for regulation of human gene expression. *Oncogene* **19**, 6194–6202.

Okudela, K., Suzuki, M., Kageyama, S., *et al.* (2007) PIK3CA mutation and amplification in human lung cancer. *Pathol. Int.* **57**, 664–671.

Oxnard, G.R., Binder, A. & Janne, P.A. (2013) New targetable oncogenes in non-small-cell lung cancer. *J. Clin. Oncol.* **31**, 1097–1104.

Petrakis, T.G., Vougas, K. & Gorgoulis, V.G. (2012) Cdc6: a multi-functional molecular switch with critical role in carcinogenesis. *Transcription* **3**, 124–129.

Sacco, E., Hasan, M.M., Alberghina, L. & Vanoni, M. (2012) Comparative analysis of the molecular mechanisms controlling the initiation of chromosomal DNA replication in yeast and in mammalian cells. *Biotechnol. Adv.* **30**, 73–98.

Sanders, H.R. & Albitar, M. (2010) Somatic mutations of signaling genes in non-small-cell lung cancer. *Cancer Genet. Cytogenet.* **203**, 7–15.

Sengupta, S., Mantha, A.K., Mitra, S. & Bhakat, K.K. (2011) Human AP endonuclease (APE1/Ref-1) and its acetylation regulate YB-1-p300 recruitment and RNA polymerase II loading in the drug-induced activation of multidrug resistance gene MDR1. *Oncogene* **30**, 482–493.

Shi, J.-H., Zheng, B., Chen, S., Ma, G.-Y. & Wen, J.-K. (2012) Retinoic acid receptor α mediates all-trans-retinoic acid-induced Klf4 gene expression by regulating Klf4 promoter activity in vascular smooth muscle cells. *J. Biol. Chem.* **287**, 10799–10811.

Shiota, M., Izumi, H., Onitsuka, T., Miyamoto, N., Kashiwagi, E., Kidani, A., Yokomizo, A., Naito, S. & Kohno, K. (2008) Twist promotes tumor cell growth through YB-1 expression. *Cancer Res.* **68**, 98–105.

Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D'Amico, M., Pestell, R. & Ben-Ze'ev, A. (1999) The cyclin D1 gene is a target of the β-catenin/LEF-1 pathway. *Proc. Natl Acad. Sci. USA* **96**, 5522–5527.

Singhal, S., Vachani, A., Antin-Ozerkis, D., Kaiser, L.R. & Albelda, S.M. (2005) Prognostic implications of cell cycle, apoptosis, and angiogenesis biomarkers in non-small cell lung cancer: a review. *Clin. Cancer Res.* **11**, 3974–3986.

Sugimura, H. (2008) Detection of chromosome changes in pathology archives: an application of microwave-assisted fluorescence in situ hybridization to human carcinogenesis studies. *Carcinogenesis* **29**, 681–687.

Sun, Y. (2006) p53 and its downstream proteins as molecular targets of cancer. *Mol. Carcinog.* **45**, 409–415.

Witzel, I.I., Koh, L.F. & Perkins, N.D. (2010) Regulation of cyclin D1 gene expression. *Biochem. Soc. Trans.* **38**, 217–222.

Wu, J., Lee, C., Yokom, D., Jiang, H., Cheang, M.C.U., Yorida, E., Turbin, D., Berquin, I.M., Mertens, P.R., Iftner, T., Gilks, C.B. & Dunn, S.E. (2006) Disruption of the Y-box binding protein-1 results in suppression of the epidermal growth factor receptor and HER-2. *Cancer Res.* **66**, 4872–4879.

Yokoyama, H., Harigae, H., Takahashi, S., Furuyama, K., Kaku, M., Yamamoto, M. & Sasaki, T. (2003) Regulation

of YB-1 gene expression by GATA transcription factors. *Biochem. Biophys. Res. Commun.* **303**, 140–145.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Figure S1 Representative samples of positive and negative findings of YB-1, cyclin D1, and CDC6.

Figure S2 Effect of YB-1 knockdown on cell proliferation and cell cycle in non-small-cell lung cancer cells.

Figure S3 Effect of YB-1 knockdown on expression of cyclin D1 protein and mRNA in non-small-cell lung cancer cells.

Table S1 Patient characteristics of NSCLCs

Impaired induction of *IL28B* and expression of *IFNλ4* associated with non-response to interferon-based therapy in chronic hepatitis C

Authors: Miyako Murakawa*[1], Yasuhiro Asahina*[1, 2], Mina Nakagawa[1], Naoya Sakamoto[3], Sayuri Nitta[1], Akiko Kusano-Kitazume[1], Takako Watanabe[1], Fukiko Kawai-Kitahata[1], Satoshi Otani[1], Miki Taniguchi[1], Fumio Goto[1], Yuki Nishimura-Sakurai[1], Yasuhiro Itsui[1], Seishin Azuma[1], Sei Kakinuma[1, 2], and Mamoru Watanabe[1]

*MM and YA contributed equally to this work.

[1]Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan

[2]Department of Liver Disease Control, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan

[3]Department of Gastroenterology and Hepatology, Hokkaido University Graduate School of Medicine, Sapporo, Japan

Correspondence: Yasuhiro Asahina, M.D., Ph.D.

Professor, Department for Liver Disease Control

Department of Gastroenterology and Hepatology

Tokyo Medical and Dental University

1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan

Tel: +81-3-5803-5877

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Fax: +81-3-5803-0268

E-mail: asahina.gast@tmd.ac.jp

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ABSTRACT

Background: Interferon (IFN) λ plays an important role in innate immunity to protect against hepatitis C viral (HCV) infection. Single nucleotide polymorphisms (SNPs) near *IL28B* (*IFNλ3*) are strongly associated with treatment response to IFNα therapy in chronic hepatitis C (CHC) patients. Recently, IFNλ4 related to *IL28B*-unfavorable allele was discovered. However, the impact of IFNλs on CHC is unknown. We aimed to investigate the mechanism underlying responsiveness to IFN-based therapy in CHC associated with SNPs near *IL28B*.

Methods: We evaluated the basal mRNA levels and ex-vivo induction of *IFNλ* expression including *IFNλ4* in peripheral blood mononuclear cells (PBMCs) from 50 CHC patients treated with PEG-IFNα/RBV. Furthermore, we investigated the effect of *IFNλ4* on induction of *IL28B* in vitro.

Results: When PBMCs were stimulated with IFNα and poly(I:C), *IL28B* induction was significantly lower in patients with *IL28B*-unfavorable genotype (rs12979860 CT/TT) than those with *IL28B*-favorable genotype (rs12979860 CC; p = 0.049). *IL28B* induction was lower in non-responders than in relapsers (p = 0.04), and it was also lower in non-SVR patients for triple therapy including NS3 protease inhibitors. *IFNλ4* mRNA was detected in 12 of 26 patients with *IL28B*-unfavorable SNP and *IFNλ4* expression was associated with lower *IL28B* induction in patients with *IL28B*-unfavorable genotype (p = 0.04) and non-response to

IFNα therapy (p = 0.003). Overexpression of *IFNλ4* suppressed *IL28B* induction and promoter activation.

Conclusions: Impaired induction of *IL28B,* related to *IFNλ4* expression in PBMCs of *IL28B*-unfavorable patients, is associated with non-response to IFNα-based therapy for HCV infection.

Keywords: hepatitis C virus, peripheral blood mononuclear cells, pegylated interferon, NS3 protease inhibitor, type III interferon

Abbreviations: HCV, Hepatitis C virus; IFN, interferon; CHC, chronic hepatitis C; PEG-, pegylated; RBV, ribavirin; DAA, direct-acting antiviral agents; SNP, single nucleotide polymorphism; IL, interleukin; TLR, Toll like receptor; RLR, RIG-I like receptor; ISG, IFN-stimulated gene; PBMC, peripheral blood mononuclear cells; SVR, sustained virological responder; VR, virological responder; NR, non-responder; poly (I:C), polyinosinic-polycytidylic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; BLC, immortalized B lymphocytes; IRF7, interferon regulatory transcription factor 7; ISRE, IFN-stimulated response element; STAT, signal transducers and activator of transcription; BDCA3, blood dendritic cell antigen 3; DC, dendric cell; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; LDL-C, low-density lipoprotein cholesterol; ISDR, IFN sensitivity determining region.

20141225

Introduction

Hepatitis C virus (HCV) infection is a common cause of chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma [1]. Interferon (IFN)-based therapy has been used to treat chronic hepatitis C (CHC) over the last two decades and the combination therapy with direct-acting antiviral agents (DAAs) improved the treating effect. However, non-responders [2] to previous pegylated interferon α (PEG-IFNα) plus ribavirin (RBV) therapy respond poorly to the triple therapy containing HCV NS3/4A serine protease inhibitors [3, 4]. Moreover, although IFN-free regimen using NS5A inhibitors or NS5B polymerase inhibitors is developed, triple or quadruple therapy including PEG-IFN may still be required to suppress DAA-resistant viruses or difficult-to-treat genotype. Therefore, IFNα responsiveness of host innate immunity remains essential for achieving a good prognosis, and determining the mechanisms responsible for non-response to IFNα is crucial.

In a recent genome-wide association study, single nucleotide polymorphisms (SNPs) located near *interleukin 28B* (*IL28B*) encoding type III IFN (IFNλ3) were found to be strongly associated with the virological response to PEG-IFNα/RBV therapy in CHC patients [5-8]. IFNλ3 is induced by viral infection through stimulation of Toll-like receptors (TLR) and RIG-I like receptors (RLR) [9-12], and it is also induced by type-I IFN signaling [13]. This interferon stimulates the expression of IFN-stimulated genes (ISGs), including numerous antiviral [13, 14] and immunoregulatory genes [15, 16]. Therefore, IFNλ3 induction may play essential roles in the innate antiviral response [17].

Recently, it was reported that high baseline expression levels of intrahepatic RLR, and lower responsiveness of ISGs to exogenous IFN, were significantly associated with unfavorable *IL28B* SNP and poor treatment outcome in CHC patients [18, 19, 20]. Furthermore, RNA sequencing using primary human hepatocytes revealed that unfavorable allele of dinucleotide polymorphisms near *IL28B* generate *IFNλ4* [21]. The ability of IFNλ4 to

20141225

induce ISGs was reported in human liver tissue samples [22]. Based on these findings, we hypothesized that preactivation of IFN signaling by *IFNλ4* prevents further induction of antiviral genes by exogenous type I IFN, particularly the type I IFN-mediated induction of IFNλs. However, the expression of *IFNλ4* has never been documented in clinical blood samples from CHC patients.

The study aimed to determine the contribution of IFNλ family (IL29 [IFNλ1], IL28A [IFNλ2], IL28B [IFNλ3] and IFNλ4) to the poor response of CHC patients to anti-HCV therapy, and to clarify the mechanisms associated with SNPs near *IL28B*. Since peripheral blood mononuclear cells (PBMCs) are major sources of IFNλ [9, 10], we measured the expression level and investigated the ex vivo induction of *IFNλs* in PBMCs derived from CHC patients receiving PEG-IFNα/RBV therapy. Furthermore, we studied the impact of *IFNλ4* on *IL28B* expression in vitro.

Methods

Patients and Clinical samples. This study included 50 CHC patients with genotype 1b HCV treated with PEG-IFNα-2b/RBV at the Tokyo Medical and Dental University Hospital. Eleven of these patients were re-treated with telaprevir (TVR) or simeprevir (SMV). Exclusion parameters were alcoholic liver injury, autoimmune hepatitis, and decompensated liver cirrhosis. No patient tested positive for hepatitis B surface antigen or anti-human immunodeficiency virus antibody, or had received immunomodulatory therapy before enrollment. The clinical characteristics of the patients immediately before blood collection are shown in Table 1.Written informed consent was obtained from all patients, and this study was approved by the ethical committee of Tokyo Medical and Dental University in accordance with the Declaration of Helsinki.

20141225

Blood samples were collected from each patient during off-therapy periods for gene expression analysis. Human genomic DNA was extracted from whole blood, and SNPs located near the *IL28B* gene (rs8099917, rs12979860, and ss469415590) were analyzed using the TaqMan SNP genotyping assay (Applied Biosystems, Carlsbad, CA) [21, 23]. HCV core mutations and IFN-stimulated response element (ISDR) substitutions were determined before the therapy.

Definitions of responsiveness to therapy. The present study used the definition of response to therapy outlined by the AASLD Practice Guideline for Diagnosis, Management, and Treatment of Hepatitis C [2, 3].

Generation of *IL28B* mRNA-specific RT-qPCR systems. For the quantification of *IL28B* mRNA expression, we developed an original real-time quantitative PCR assay that distinguishes *IL28B* from *IL28A*. Gene-specific PCR primers were designed to anneal directly to the cDNA sequences of each gene (Supplementary Table 1).

Real-time detection RT-PCR analysis for *IL28A*, *IL28B*, and *IL29*. Immediately after blood collection, PBMCs were separated by gradient centrifugation with Ficoll-Conray, and incubated in the RPMI 1640 medium (Sigma, St. Louis, MO) with 10% fetal calf serum at 37°C under 5% CO_2. The cells were treated with recombinant IFNα-2b (100 IU/ml) (Schering-Plough, Kenilworth, NJ) for 12 h prior to polyinosinic-polycytidylic acid (poly(I:C)) (Sigma) treatment (10 μg/ml) for 8 h. PBMC RNA was extracted using the RNeasy Mini Kit (Quiagen, Valencia, CA). Total cell RNA (200 ng) was used to generate 10 μl of cDNA from each sample using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). The mRNA expression levels were measured using a ABI 7500 real-time PCR system (Applied Biosystems), and a QuantiTect SYBR Green PCR kit (Quiagen) or TaqMan Universal PCR Master Mix (Applied Biosystems). Expression levels were normalized to the

expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin. The sequences of the primer sets are provided in Supplementary Table 1.

Analysis of *IFNλ4* mRNA expression. Total cell RNA was pre-treated with DNase-I (Nippon Gene, Tokyo, Japan), followed by RT with SuperScript II, and PCR analysis was performed for 45 cycles using 4 sets of primers (Supplementary Table 1). Primer set #1 could detect 1 copy of IFNλ4 per assay, whereas primer sets #2, 3, and 4 could detect 10 copies of IFNλ4 per assay (Supplementary Fig. 1). The PCR products corresponding to the size of spliced *IFNλ4* mRNA were extracted and the sequences were confirmed. Only the amplicon with ss469415590-ΔG (*) is defined as IFNλ4 (Supplementary Figs. 1, 2).

Generation of the *IL28B* promoter-reporter and stably expressing cell lines. The promoter sequences of human *IL28B* (-1129/+111) were subcloned and the DNA fragment was inserted into the pGL3-basic vector (Invitrogen). The reporter plasmid was transfected into HEK293 cells with pcDNA3.1 (Invitrogen). After cell culture in the presence of the selective antibiotic G418 (Nacalai Tesque, Kyoto, Japan), transfected colonies were isolated to establish a cell line stably expressing the IL28B-Fluc-reporter (HEK293/ IL28B-luc).

Cell culture. HEK293T, Huh7, HepG2, and HeLa cells were maintained in Dulbecco's modified Eagle's Medium (Sigma) supplemented with 10% fetal calf serum (37°C; 5% CO_2). The maintenance medium for the IL28B-promoter-reporter-harboring cell line (HEK293/IL28B-luc) was supplemented with 500 μg/ml of G418 (Nacalai Tesque). Immortalized B lymphocytes (BLC) were generated in-house from human PBMCs by EBV transformation, and maintained in RPMI1640 medium (Sigma) with 10% feral calf serum and 200 ng/ml Cyclosporin-A (Sigma). The HuS/E-2 cells were kindly provided by Dr. Hijikata (Kyoto University, Kyoto, Japan) and cultured as previously described [24].

Expression plasmids and transfections. The expression construct for IFNλ4 (p179) was kindly provided by Dr. Prokunina-Olsson (National Cancer Institute, Bethesda, MD). The

DNA fragments of IRF7 were inserted into the vector pcDNA4/TO/myc-His (Invitrogen). The expression plasmids for p50 and p65 were kindly provided by Dr. Rongtuan Lin (Lady Davis Institute for Medical Research, Baltimore, MD). pcDNA4/TO/myc-His vector (Invitrogen) was used as control for mock transfection.

BLC were transfected with *IFNλ4* plasmids or control plasmids by electroporation using Gene Pulser Xcell Electroporation System (BIO RAD, Hercules, CA). After 24 h, cells were treated with mock, recombinant IFNα-2b (100 IU/ml) (Schering-Plough) for 24h. *IFNλ4* plasmids and *IRF7* plasmids or control plasmids were co-transfected into HEK293T cells with Lipofectamine LTX reagent (Invitrogen) and Opti-MEM medium, according to the manufacturer's instructions. Total RNA was extracted and quantified by real-time qRT-PCR.

Luciferase assays. *IFNλ4* or control plasmids were transfected into HEK293/IL28B-luc cells and the cells were treated with IFNα for 24h next day. HEK293/IL28B-luc cells were co-transfected with IFNλ4 plasmids and IRF7, p50: p65 or control plasmids and incubated for 24h. MTS viability and single luciferase assays were conducted by 1420 Multilabel Counter (ARVO MX, PerkinElmer, Boston, MA) using a CellTiter 96 AQueous One Solution System (Promega, Madison, WI) and a Bright-Glo Luciferase Assay System (Promega), as previously described [25, 26].

Statistical analyses. The data were analyzed using the Welch's *t* test for continuous variables and the chi-square test for categorical data. p values < 0.05 were considered statistically significant.

Results

Genotype of *IL28B* SNP and expression of *IL29*, *IL28A*, and *IL28B* mRNA in PBMC. Three SNPs near the *IL28B* gene (rs8099917, rs12979860, and ss469415590) were genotyped. The number of patients with each genotype is shown in Table 1. In agreement with a recent

report from the HapMap Project in Asia [21], the genotype of ss469415590 was completely correlated with that of rs12979860 in this study, while 3 of 50 patients have different genotype between ss469415590 and rs8099917. Baseline mRNA expression levels of *IL29*, *IL28A*, and *IL28B* were not influenced by the rs12979860 genotype (Fig. 1A). However, when PBMCs were stimulated with IFNα and poly(I:C), the induction of *IL28B* expression was significantly lower in patients with the *IL28B*-unfavorable genotype (rs12979860 CT/TT) than in those without (rs12979860 CC) ($p = 0.049$) (Fig. 1B).

Relationship of therapy response with *IL29*, *IL28A*, and *IL28B* mRNA levels in PBMC. We assessed the relationship between the expression level of the *IFNλs* and the virological response to PEG-IFNα/RBV therapy. At baseline, there was no significant difference in *IFNλs* expression between the SVR, relapser, and NR patients (data not shown). On the other hand, the induction of *IL28B* expression by IFNα and poly(I:C) decreased with the patients' response to therapy (Fig. 2A). The mRNA levels of NR patients were significantly lower than those for relapsers ($p = 0.04$) as well as VR ($p=0.005$). The induction of IL29 expression of NR patients were lower than those for VR ($p=0.048$). In contrast, the induction of *IL28A* did not reveal any association between mRNA levels and treatment response.

When the *IL28B* induction levels of VR and NR patients were further stratified by genotype, it was significantly lower in NR than in VR patients in both rs12979860 CC and CT/TT subgroups ($p = 0.01$ and 0.02, respectively) (Fig. 2B).

Furthermore, 11 of 32 non-SVR patients were re-treated with NS3 protease inhibitor (TVR or SMV) plus PEG-IFNα/RBV triple therapy; 2 of them were IL28B-favorable and 9 were unfavorable. Even treated with NS3 protease inhibitor, it should be noted that IL28B inductions in non-SVR of the triple therapy were significantly lower than those in SVR of the PEG-IFNα/RBV therapy or triple therapy ($p=0.017$). IL28B inductions in non-SVR were also

lower than those in SVR of triple therapy (3.5 vs 12.1 fold induction). IL28A inductions in non-SVR of the triple therapy were also significantly lower than those in SVR (p=0.042) (Fig. 2C).

Impact of *IL28B* genotype and induction on *IFNλ4* mRNA expression. We measured the expression level of *IFNλ4* in PBMCs derived from CHC patients. Because we could not detect *IFNλ4* mRNA in PBMCs with RNA sequencing nor the previously reported TaqMan real-time quantitative RT-PCR system [21], we designed a new highly sensitive RT-PCR system using 4 sets of primers. The detection threshold was as low as 1–10 copies/assay (Supplementary Table 1; Supplementary Fig. 1A). This RT-PCR assay allowed us to confirm the full length mRNA sequence of *IFNλ4* in poly(I:C)-treated HepG2, HeLa, HEK293T cells, and BLC from ss469415590-ΔG/ΔG patients by amplicon sequencing (Supplementary Figs. 1B,C).

Using this system, we tested PBMCs from 47 CHC patients for the presence of *IFNλ4* mRNA. Among the 23 patients with *IL28B*-unfavorable rs12979860 [T] and ss469415590 [ΔG]-allele, *IFNλ4* mRNA was detected in 12 patients (7 in non-stimulated PBMCs and 8 in IFN-poly(I:C)-stimulated PBMCs). In marked contrast, *IFNλ4* mRNA was not detected in any of the *IL28B*-favorable patients (Supplementary Fig. 2). There was no significant difference in baseline expression of *IFNλ*s between patients with or without detectable *IFNλ4* expression (Fig. 3A). However, the induction of *IL28B* expression by IFN-poly(I:C) was significantly lower in patients with *IFNλ4* mRNA than those without detectable *IFNλ4* ($p = 0.008$) (Fig. 3B). Even among *IL28B*-unfavorable patients (rs12979860 CT/TT), *IL28B* induction levels were significantly lower in *IFNλ4*-positive patients ($p = 0.04$) (Fig. 3B). Although induction of *IL28A* was lower in *IFNλ4*-positive patients than *IFNλ4*-negative patients ($p = 0.04$), there was no significant relation between *IFNλ4* expression and the induction of *IL28A* and *IL29* among *IL28B*-favorable patients.

Association between *IFNλ4* expression and clinical response to antiviral therapy. The rate of virological non-response was significantly higher in patients with *IFNλ4* mRNA than in all those without detectable *IFNλ4* ($p = 0.003$; Fig. 3C). Among the *IL28B*-unfavorable patients (rs12979860 CT/TT), the virological non-response rate also tended to be higher in patients expressing *IFNλ4* ($p = 0.08$).

Suppression of *IL28B* induction by *IFNλ4* in vitro. The mechanism behind the lower induction of *IL28B* mRNA in CHC patients expressing *IFNλ4* was investigated by testing whether the expression of *IL28B* is influenced by overexpression of *IFNλ4* in vitro. When IFNλ4 was overexpressed, baseline expression of IL28B was significantly increased in HEK293, BLC (Supplementary Fig. 3). However, as shown in Fig. 4A, IL28B expression was increased by IFNα (1.8 fold induction, p=0.012) but that induction was suppressed in the presence of IFNλ4 (1.2 fold induction, p=0.28) in BLC. As *IL28B* promoter is known to be activated by the transcription factors such as IRF7 and NFκB [11, 31], we next evaluated IL28B induction by IRF7. *IL28B* mRNA was induced by IRF7 in dose dependent manner and the induction levels were suppressed by *IFNλ4* overexpression significantly (Fig. 4B). *IL28B* promoter activities induced by IFNα, IRF7 and p50:p65 were also inhibited by *IFNλ4* overexpression (Fig. 4C-E).

Discussion

The present study shows that the inducibility of *IL28B* expression is associated with virological responsiveness to IFNα in CHC patients, and it is also related to the *IL28B* genotype. Furthermore, we detected *IFNλ4* mRNA in PBMCs using an original sensitive RT-PCR system. *IFNλ4* suppressed *IL28B* induction and associated with virological non-responses to IFNα-based antiviral therapy.

20141225