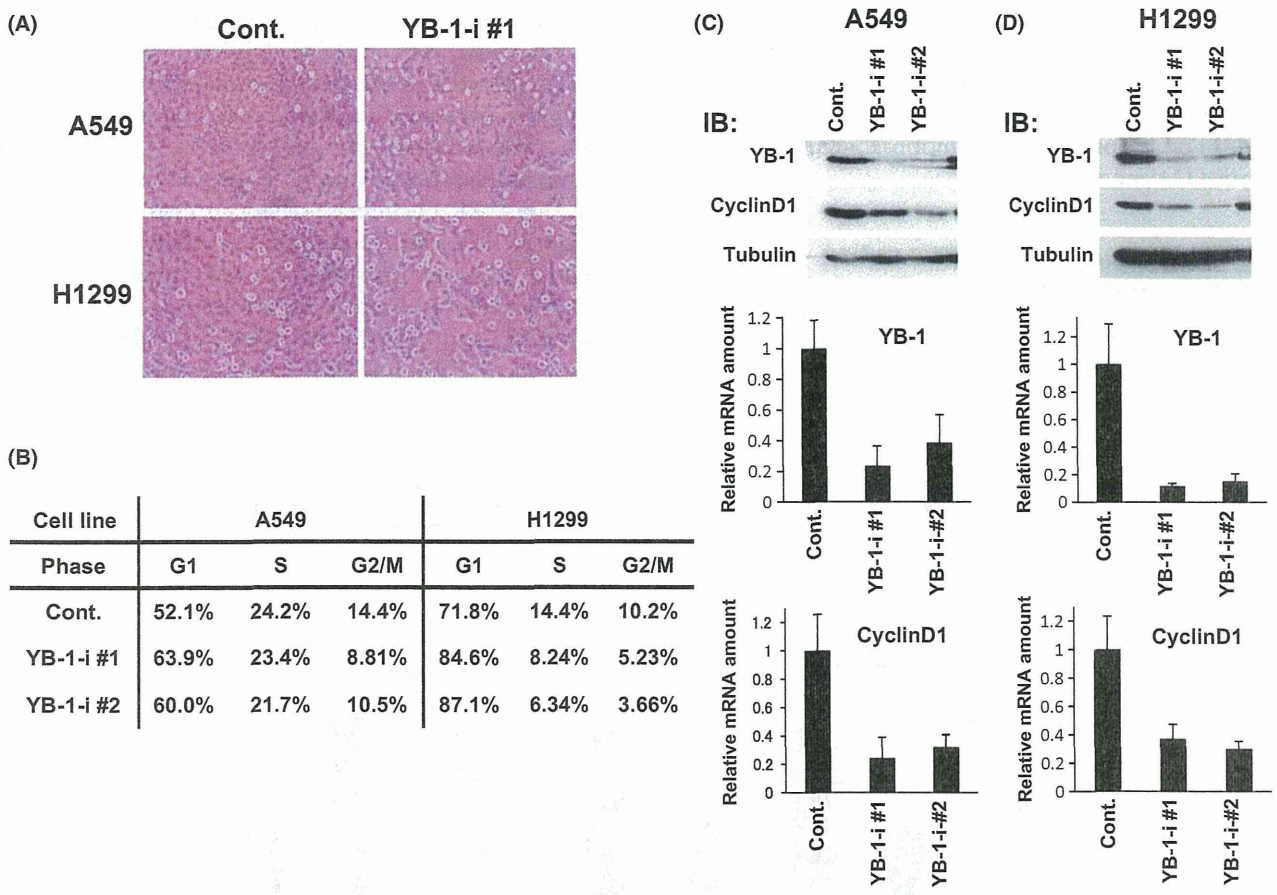

(B)

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*

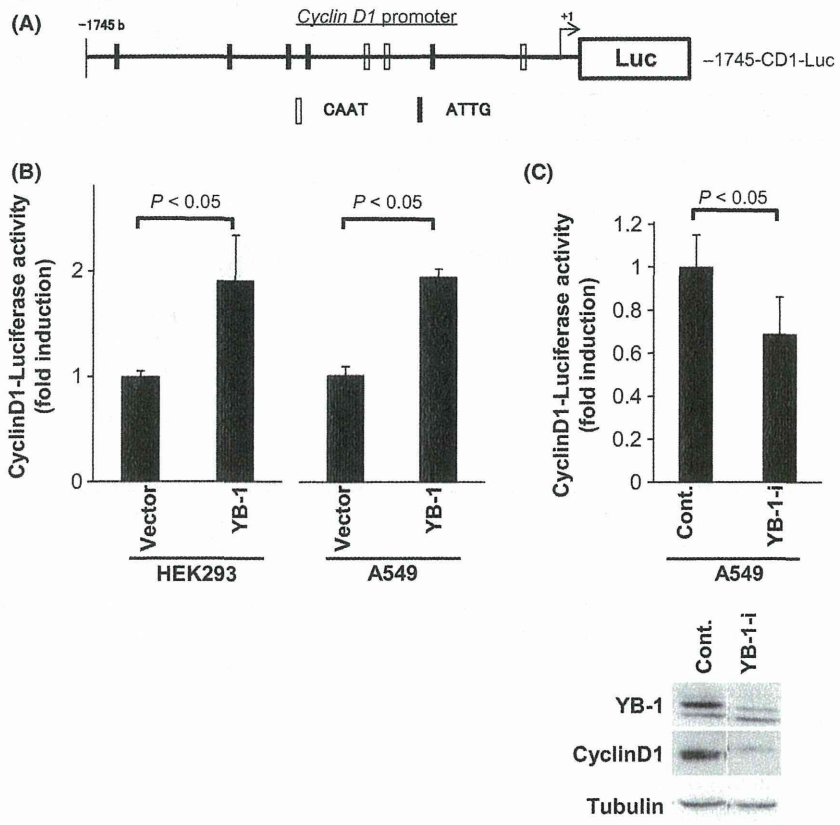

Figure 2 YB-1 enhanced the transcriptional activity of cyclin D1. (A) Schematic representation of the *cyclin D1* 5′ promoter construct in the pSPLUC vector. Potential YB-1-binding sites (Y-boxes), ATTG (black box) or CAAT (open box), are shown. (B) Luciferase assays using the *cyclin D1* reporter in 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

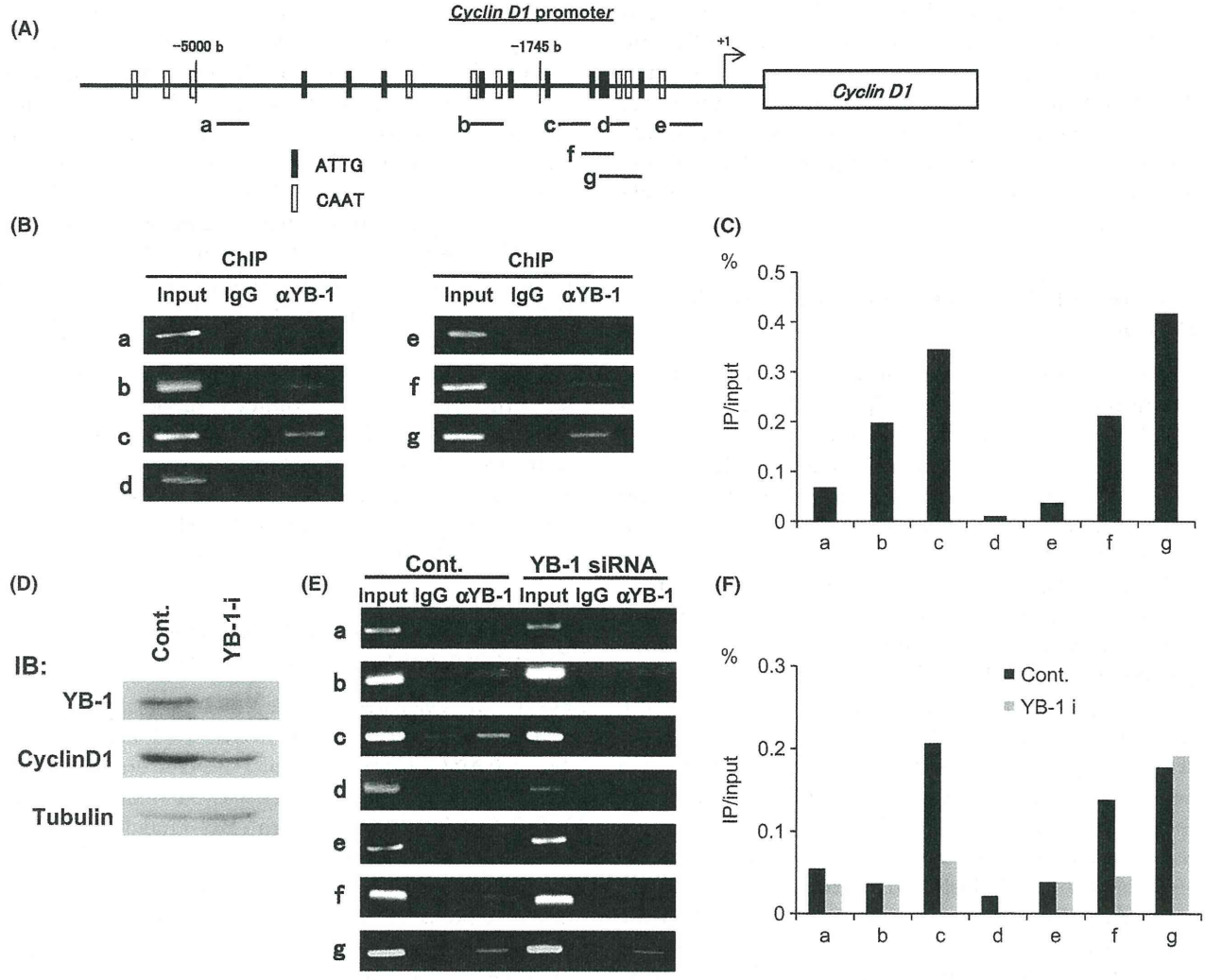

Figure 3 YB-1 directly binds the promoter region of cyclin D1. (A) Schematic representation of the potential YB-1 binding sites and the location of the primers used for chromatin immunoprecipitation (ChIP) in the promoter region of cyclin D1. Black bars (a–g) show regions for PCR primers. Black and open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

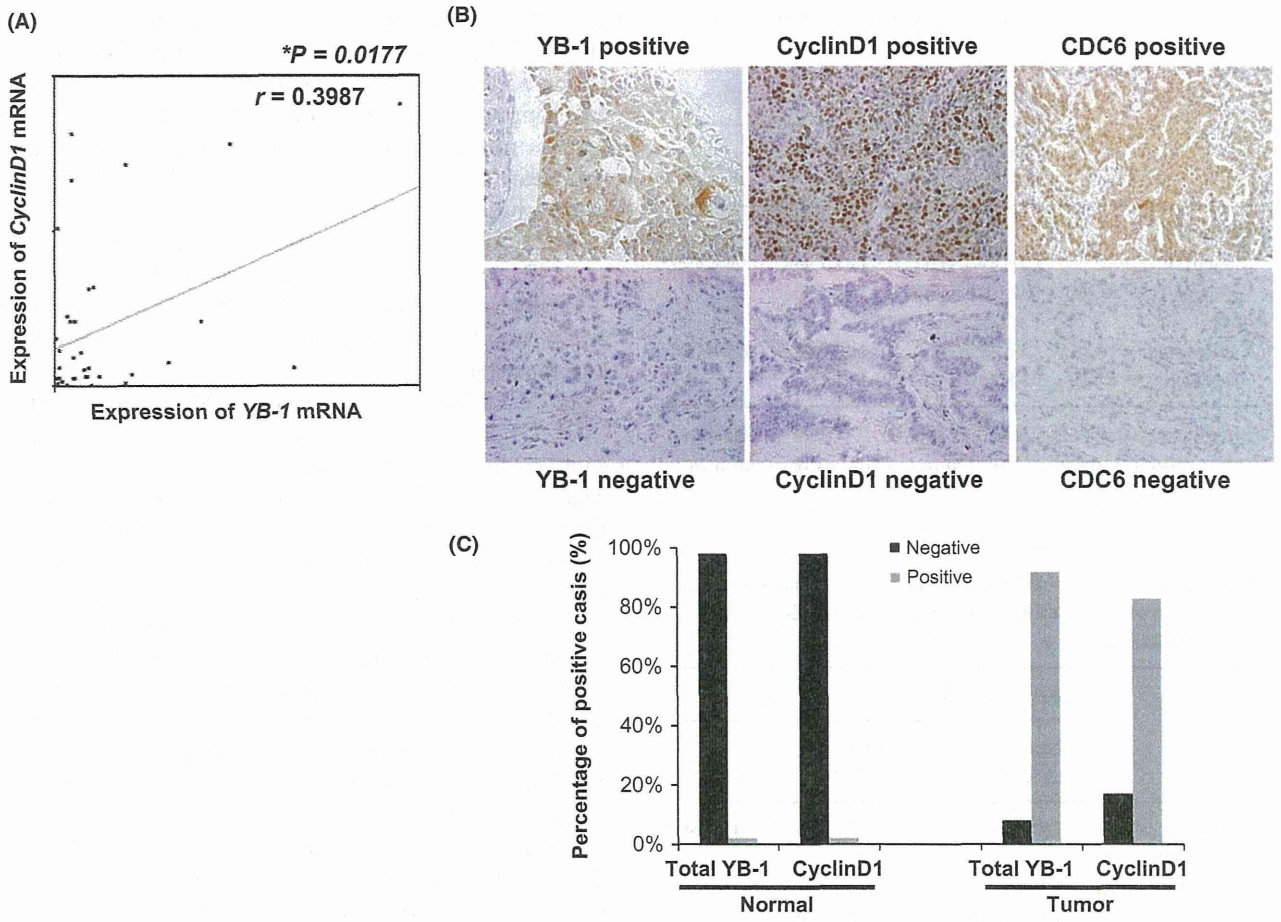

Figure 4 YB-1 and cyclin D1 mRNA and protein expression in non-small-cell lung cancer (NSCLC) clinical samples. (A) The expression of YB-1 and cyclin D1 mRNA was analyzed using RT-qPCR in 34 patients with NSCLC. To determine whether there is a positive correlation between total mRNA expression of YB-1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

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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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

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Short Report

Ethnic differences in *GRHPR* mutations in patients with primary hyperoxaluria type 2

Takayama T, Takaoka N, Nagata M, Johnin K, Okada Y, Tanaka S, Kawamura M, Inokuchi T, Ohse M, Kuhara T, Tanioka F, Yamada H, Sugimura H, Ozono S. Ethnic differences in *GRHPR* mutations in patients with primary hyperoxaluria type 2.
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The objective of this study was to investigate ethnic differences in the glyoxylate reductase/hydroxypyruvate reductase (*GRHPR*) gene in patients with primary hyperoxaluria type 2 (PH2). *GRHPR* was genotyped in Japanese patients with PH2 and all *GRHPR* mutations described to date were reviewed in terms of geographic and ethnic association. We identified a novel mutation, a two-nucleotide deletion (c.248_249delTG) in exon 3 creating a premature 'stop' at codon 91. Also, we found that the c.864_865delTG mutation was associated with the rs35891798 single-nucleotide polymorphism. The allelic frequencies of the c.103delG, c.494G>A, c.403_404+2 delAAGT, and c.864_865delTG mutations in PH2 patients were 37.8%, 15.6%, 10.0%, and 10.0%, respectively. All patients with the c.103delG mutation were Caucasian. Patients with the c.494G>A mutation and 78% (7/9) of those with the c.403_404+2 delAAGT mutation were from the Indian subcontinent, whereas those with the c.864_865delTG mutation were Chinese or Japanese. Molecular analysis of *GRHPR* of four Japanese PH2 patients identified a novel mutation (c.248_249delTG in exon 3). Caucasians with PH2 should be screened for the c.103delG mutation; patients from the Indian subcontinent for c.494G>A; and patients of East Asian origin (particularly) for c.864_865delTG. The prevalence of the latter mutation in PH2 patients from East Asia was 75.0%.

Conflict of interest

None.

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Key words: ethnic – *GRHPR* – mutation – primary hyperoxaluria type 2

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Primary hyperoxaluria type 2 (PH2) is a rare inherited autosomal recessive disorder characterized by excessive levels of urinary oxalate and L-glycerate. PH2 is caused by mutations in the *GRHPR* gene encoding glyoxylate/hydroxypyruvate reductase (1). We previously developed a diagnostic algorithm (1) useful for the diagnosis of PH2. Candidate patients present with nephrocalcinosis and/or calcium oxalate stones, or with a family history of PH. Pediatric patients who meet one or more of these criteria are high priorities for

evaluation. Patients exhibiting high-level 24-h urinary oxalate excretion or elevated plasma oxalate concentrations (except those with secondary hyperoxaluria) receive a preliminary diagnosis of PH. Next, urinary L-glycerate and glycolate levels are measured, as GRHPR activity in blood monocytes (2). Mutation analysis follows. Lack of GRHPR activity in monocytes or liver biopsy samples, associated with elevated urinary oxalate and L-glycerate levels, allows a definitive diagnosis of PH2. Such diagnosis may be verified by genetic analysis of *GRHPR*. However, liver biopsy is of course invasive and unattractive to patients.

Currently, the National Center for Biotechnology Information for single-nucleotide polymorphism (SNP) lists at least 37 missense mutations in *GRHPR* and it has been reported that 15 mutations in *GRHPR* cause loss of enzyme expression or function (3–10). Previous studies suggested that allelic frequency varies among different PH2 populations. The most common mutation is c.103delG in exon 2, which creates a frameshift inducing premature termination at codon 451. This mutation constitutes about 40% (3, 6) of all known mutations causing PH2, and appears to have originated in a founder of Northern European or American origin (7).

In Japan, the prevalence of both primary hyperoxaluria type 1 (PH1) and PH2 is poorly known, although we reviewed literature data on 59 Japanese PH1 patients described between 1962 and 2003 (11) and reported on a single PH2 patient (9). Recently, primary hyperoxaluria type 3 (PH3; OMIM 613616) has been identified in some unclassified PH patients; this condition is characterized by mutations in the gene (*HOGA1*) encoding 4-hydroxy-2-oxoglutarate aldolase; the gene was formerly termed *DHDPSL* (12). The frequencies of mutations causing PH2 and PH3 have not been described in Asian patients. In this report, we describe the genetic features of four Japanese patients with PH2 and the ethnic distribution of *GRHPR* variants.

Materials and methods

Patient profiles are shown in Table 1. Four patients were diagnosed with PH2 using gas chromatography/mass spectrometry-based urine metabolome analysis (13). No liver biopsies were performed. Genomic DNAs from the four patients; from both parents of Patients 1, 2, 4, and 5; and from 14 patients with recurrent calcium oxalate urolithiasis but without hyperoxaluria were extracted from peripheral blood samples using QIAamp kits (Qiagen, Hamburg, Germany) according to the manufacturer's instructions. Informed consent was obtained from all patients and their parents in line with the requirements of the Institutional Review Board of Hamamatsu University School of Medicine. Polymerase chain reaction (PCR) was used to explore all splicing acceptor and donor sites using a modification (14) of a previously described method (4, 9). DNAs were sequenced on ABI Prism 377 or ABI 310 DNA platforms and the sequences were compared with the known human *GRHPR* sequence (GenBank accession no. NM_012203) using NCBI BLAST alignment.

Table 1. Clinical features of Japanese patients with primary hyperoxaluria type 2

Patient/ Gender	Age at symptom development	Symptom	Renal parenthymal calcifications	Age at diagnosis	Location of stones at diagnosis	Urinary excretion levels		Present age	Renal function	Clinical course
						Oxalate	Glycerate			
1/Male	10 months	UTI	–	7 years 2 months	Bilateral kidney	1.9, 1.8 SD	386, 473 mmol/mol Cr	7 years	WNL	SWL, 9 times
2/Male	3 years 10 months	Gross hematuria	–	4 years 8 months	Bladder	1.7, 2.2 SD	612, 587 mmol/mol Cr	25 years	WNL	Lithotripsy, twice SWL,once SWL, 5 times
3[a]/Male	7 months	Gross hematuria	–	10 months	Bilateral kidney and ureter	227±120 (mg/day/1.73 m^2)	3,032 ± 1,276 (mg/day/1.73 m^2)	20 years	WNL	
4/Male	3 years	Gross hematuria	–	21 years	Bilateral kidney	0.2 mg/mg Cr	504 mmol/mol Cr	21 years	WNL	–
5/Female	2 years	Gross hematuria	+	8 years	Bilateral kidney	1.69 mg/mg Cr	1,081 mmol/mol Cr	8 years	WNL	–

SD, standard deviation; SWL, shock wave lithotripsy; UTI, urinary tract infection; WNL, within normal limits.
[a]The details of Patient 3 have been reported in Ref. (8). Urinary excretion levels of oxalate and glycerate by Patients 1 and 2 were measured in the Division of Human Genetics, Medical Research Institute, Kanazawa Medical University, using the technique of Ref. (5). Two and three SDs of the measured glycerate levels was 7.8 and 16.6 mmol/mol Cr, respectively. Urinary excretion levels of oxalate and L-glycerate by Patients 4 and 5 (siblings) were measured in the Research Institute of Medical Mass Spectrometry, Kurume University School of Medicine, using a modification of the technique of Ref. (5). Mean ± 3SD of L-glycerate levels was 168.5 mmol/mol Cr.

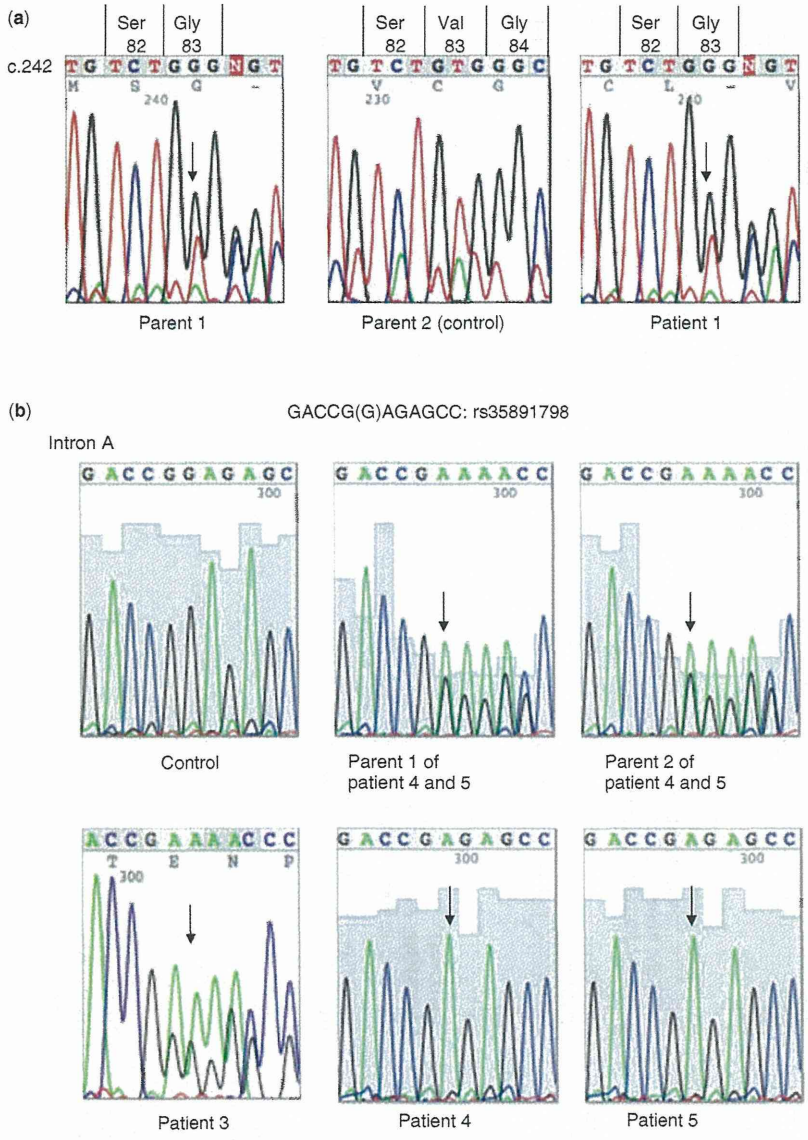

Fig. 1. Nucleotide sequences of mutated exons and introns. Isolated sections of electropherograms are shown. The deduced nucleotide sequence and *GRHPR* codons are shown above each panel. Arrows: affected nucleotide(s). All electropherograms show the forward sequence. (**a**) Electropherogram of exon 3 of Patient 1. (**b**) Electropherograms of intron A of Patients 3, 4, and 5, and the parents of Patients 4 and 5.

All published data on PH2 were reviewed. We incorporated information gained from personal communications. We also included our new *GRHPR* sequence data. The two criteria used to explore ethnic associations between *AGT* mutations and PH1 (15) were employed to explore whether particular *GRHPR* mutations might exhibit such associations. The criteria were, first, that a relevant mutation had to be largely confined to one population group or geographic area; and, second, that the mutation was associated with PH2 in at least two unrelated individuals.

To obtain the background prevalence of these genotypes in unrelated Japanese individuals, we designed Taqman^R probes to identify these genotypes in totally 237 individuals from the previous cohort in the rural city in the same prefecture (n = 125) (16) and unrelated outpatients (n = 112) without history of urolithiasis in our department.

Briefly, these DNAs were extracted from the blood samples given by the participants using a QIAamp DNA Blood Maxi kit according to the manufacturers' instructions (Qiagen). A 50 ng sample of each subjects DNA was amplified by PCR, with the primer set for using custom Taqman^R probe identifying *GRHPR* Ex8 (c.864_865del GT) and *GRHPR* intron 1 (c.83+51delG; rs35891798) by using the StepOne (Applied Biosystems, Carlsbad, CA). The Assay IDs above (Assay Names) are AH89ZKB (PH2_0001) and AHABEP4, respectively.

Table 2. The GRHPR gene mutations[a]

Exon/Intron	Nucleotide	cDNA change	Amino acid change	Allelic frequency							
				Cramer et al. (3) and Webster et al. (7, 8)	Cregeen et al. (4)	Lam et al. (6)	Johnson et al. (5)	Levin-laina et al. (10)	Our cases (9)	Total	%
Intron A	c.84-2A>G	Splicing error	n/a		3					3	3.3
	c.84-13_c.84-12del; c.84-8_c.84-5del	Splicing error	n/a		1					1	1.1
Exon 2	c.103delG	1 bp deletion	Asp35Thr; frameshift, aa 44X	18[b]	14		2			34	37.8
Exon 3	c.248_249delTG[c]	2 bp deletion	Val83Gly; frameshift, aa 91X						1	1	1.1
Exon 4	c.295C>T	Nonsense transition	Arg99X	4	2					6	6.7
	c.337G>A	Missense transition	Glu113Lys						1	1	1.1
	c.375delG	1 bp deletion	Leu126Cys; frameshift, aa 133X		1					1	1.1
Exon 4/Intron D	c.403_405+2 delAAGT	4 bp deletion and splicing error	n/a	2	7					9	10.0
Exon 6	c.494G>A	Missense transition	Gly165Asp	3[d]	5		6			14	15.6
	c.540delT	1 bp deletion	Leu181Cys; frameshift, aa 204X		1					1	1.1
Exon 7	c.608_609delCT	2 bp deletion	Pro204Leu; frameshift, aa 210X		3					3	3.3
Intron G	c.246-2A>G	Missense transition and splicing error	n/a	1						1	1.1
Exon 8	c.864_865delTG	2 bp deletion	Val289Asp; frameshift, aa 310X			2			7	9	10.0
Exon 9	c.904C>T	Missense transition and splicing error	Arg302Cys		1				1	2	2.2
	c.934A>G	Missense transition	Asn312Asp					2		2	2.2
	c.965T>G	Missense transversion	Met322Arg	2						2	2.2
				30	38	2	8	2	10	90	100.0

a/a, amino acid; n/a, not analyzed; X, stop codon.

[a]Sixteen mutations in all 90 alleles including two PH2 patients whom we newly performed genotyping and four those with personal communication by Dr Scott Cramer.

[b]Three homozygous patients are provided by personal communication with Dr Scott Cramer.

[c]This is a novel mutations we detected in this report.

[d]One homozygous patient is provided by personal communication with Dr Scott Cramer.

Table 3. The relationship between the mutation and ethnic origin in 45 patients with primary hyperoxaluria type 2

No. of patients	Ethnic origin	Geographic area	Allelic mutations		Authors
1	Japanese	East Asia	c.248_249delTG[a]	c.904C>T	
2	Japanese	East Asia	c.864_865delTG	c.864_865delTG	
3[b]	Japanese	East Asia	c.337G>A	c.864_865delTG	Our cases (9)
4	Japanese	East Asia	c.864_865delTG	c.864_865delTG	
5	Japanese	East Asia	c.864_865delTG	c.864_865delTG	
6	Caucasian American	America	c.103delG	c.103delG	
7	Caucasian American	America	c.103delG	c.103delG	Cramer et al. (3)
8	Caucasian American	America	c.103delG	c.103delG	
9	Caucasian American	America	c.103delG	c.103delG	
10	Caucasian Italian	Europe	c.295C>T	c.295C>T	
11	Caucasian Italian	Europe	c.295C>T	c.295C>T	
12	Caucasian Italian	Europe	c.403_404+2 delAAGT	c.403_404+2 delAAGT	
13	Indian subcontinent	Mid East	c.494G>A	c.246-2A>G	Webster et al. (7, 8)
14	Caucasian American	America	c.103delG	c.103delG	
15	African American	America	c.965T>G	c.965T>G	
16	Caucasian German	Europe	c.103delG	c.103delG	
17	Caucasian American	America	c.103delG	c.103delG	
18	Caucasian American	America	c.103delG	c.103delG	Dr Scott Cramer,
19	Caucasian American	America	c.103delG	c.103delG	Personal communication
20	Indian subcontinent (Arabic)	Mid East	c.494G>A	c.494G>A	
21	Caucasian	Europe	c.84-2A>G	c.84-2A>G	
22	Caucasian	Europe	c.103delG	c.84-2A>G	
23	Caucasian	Europe	c.103delG	c.103delG	
24	Caucasian	Europe	c.103delG	c.103delG	
25	Caucasian	Europe	c.103delG	c.103delG	
26	Caucasian	Europe	c.103delG	c.103delG	
27	Caucasian	Europe	c.103delG	c.103delG	
28	Caucasian	Europe	c.103delG	c.103delG	
29	Caucasian	Europe	c.103delG	c.904C>T	Cregeen et al. (4) and Dr
30	Caucasian	Europe	c.295C>T	c.295C>T	Gill Rumsby, Personal
31	Caucasian	Europe	c.375delG	c.608-609_delCT	communication
32	Caucasian	Europe	c.608-609_delCT	c.608-609_delCT	
33	Indian subcontinent	Mid East	c.494G>A	c.494G>A	
34	Indian subcontinent	Mid East	c.494G>A	c.494G>A	
35	Indian subcontinent	Mid East	c.403_404+2 delAAGT	c.403_404+2 delAAGT	
36	Indian subcontinent	Mid East	c.403_404+2 delAAGT	c.403_404+2 delAAGT	
37	Indian subcontinent	Mid East	c.403_404+2 delAAGT	c.403_404+2 delAAGT	
38	Indian subcontinent	Mid East	c.403_404+2 delAAGT	c.540delT	
39	Indian subcontinent	Mid East	c.494G>A	c.84-13_c.84-5delinsCTTT	
40	Chinese	East Asia	c.864_865delTG	c.864_865delTG	Lam et al. (6)
41	Indian subcontinent (Afghanistan)	Mid East	c.494G>A	c.494G>A	
42	Indian subcontinent (Afghanistan)	Mid East	c.494G>A	c.494G>A	Johnson et al. (5)
43	Indian subcontinent (Afghanistan)	Mid East	c.494G>A	c.494G>A	
44	Caucasian England	Europe	c.103delG	c.103delG	
45	Yemenite	Mid East	c.934A>G	c.934A>G	Levin-Iaina et al. (10)

[a]Novel mutations.
[b]This patient was reported in Ref. (8).

Written informed consents were obtained from all the participants and the study design was approved by Institutional Review Board of Hamamatsu University School of Medicine.

Results

Table 1 shows the clinical features of Japanese patients with PH2. We identified a novel *GRHPR* mutation associated with this disease. In Patient 1, a compound heterozygote for the *GRHPR* gene, we found a two-nucleotide deletion (c.248_249delTG) in exon 3 which resulted in premature termination at codon 91 (Fig. 1a). We also found a known missense transition, c.904C>T (Arg302Cys) in exon 9, which causes GRHPR activity to be only 5.6% that of the wild-type control (5). Patients 2, 4, and 5 were homozygotic for the c.864_865delTG mutation. Moreover, a SNP in intron A (rs35891798) was identified in Patient 3 (a heterozygote) (8); in the parents (heterozygotes) of Patients 2, 4 and 5; and in Patients 2, 4, and 5 (homozygotes) (Fig. 1b and Figure S1b,c, Supporting Information). The c.864_865delTG mutation was associated with the rs35891798 SNP. However, in Patient 1 and the parents of that patient (Figure S1a), and in 14 patients with recurrent urolithiasis in the absence of hyperoxaluria, c.864_865delTG was not associated with the rs35891798 SNP (data not shown).

Among the unrelated Japanese, there were no mutant allele of *GRHPR* c.864_865delTG (Figure S2a)(0/474 alleles; a DNA from one subject did not generate the clear genotype result). Nine heterozygous and one homozygous subjects were identified as a polymorphism in the intron 1, rs35891798.

Table 2 lists known *GRHPR* mutations and Table 3 shows relationship between PH2-causing mutations and patient ethnic origin; information derived from personal communications with Drs Scott Cramer and Gill Rumsby is included. The allelic frequencies of the c.103delG, c.494G>A, c.403_404+2delAAGT, and c.864_865delTG mutations were 37.8%, 15.6%, 10.0% and 10.0%, respectively. All patients with the c.103delG or c.295C>T mutations were Caucasian (European or American); all those with the c.494G>A mutation and 78% (7/9 alleles) of those with the c.403_404+2 delAAGT mutation were from the Indian subcontinent; and all patients with the c.864_865delTG mutation were Chinese or Japanese.

Sixteen *GRHPR* mutations have now been described, one of which is newly reported here. The mutations occur all along the *GRHPR* gene, with the exception of exon 1, and 44% are single-nucleotide substitutions (seven missense and one nonsense); eight small deletions have also been described.

Discussion

We explored *GRHPR* mutations in four patients diagnosed with PH2 with the aid of gas chromatography/mass spectrometry-based urine metabolome analysis (13); we did not perform liver biopsies. We found a novel mutation, c.248_249delTG, in exon 3, and also showed that the c.864_865delTG mutation in exon 8 was linked to the rs35891798 SNP of intron A. Moreover, we determined that the c.864_865delTG mutation was of Chinese or Japanese origin.

In terms of the diagnosis of primary hyperoxaluria, Rumsby et al. (17) indicated that genetic screening afforded definitive diagnosis in 34% of PH1 patients and 33% of those with PH2. In that report, however, the only candidate mutation for PH2 was c.103delG. In this study, we found a relationship between *GRHPR* mutations and ethnic distribution. The c.103delG mutation is found only in Caucasians, but c.494G>A and 78% (7/9) of c.403_404+2delAAGT mutations occur in patients of the Indian subcontinent (one such patient was an Italian Caucasian). The c.864_865delTG mutation occurs only in Chinese and Japanese. The allelic frequency of the c.864_865delTG mutation in three Japanese and one Chinese PH2 patient was 75% (9/12). Thus, our data will be of use when genetic screening for PH2 is planned, and may obviate the need for invasive biopsy.

The association between the c.864_865delTG mutation in exon 8 and the rs35891798 SNP in intron A is particularly noteworthy. As the c. 864_865delTG mutation is located toward the end of exon 8, the mutation may be associated with translational difficulty. If the observed linkage is indeed robust, diagnosis of PH2 would be facilitated. The linkage appeared to be absent in patient 1 (without the c. 864_865delTG mutation) and in 14 patients with recurrent urolithiasis but without hyperoxaluria. It is necessary to explore the frequency of the rs35891798 SNP in large number of healthy subjects with detailed information on urolithiasis and life styles including known and unknown risk factors. Our preliminary small scale survey on 230 subjects revealed 11 alleles of 460 alleles were mutants. Although detailed urological information is not available for the homozygous subject on rs35891798, the questionnaire he filled in at the interview did not include urolithial problem as the past history. The absence of c.864_865delTG mutant alleles in this unrelated Japanese, apparently not symptomatic on urolithiasis strengthens our interpretation that this functional mutation is responsible for PH2 in Japanese.

The number of PH2 patients is small compared to those with PH1. To date, only 16 mutations in *GRHPR* have been described, one of which is newly reported here. Although the *AGXT* mutations are predominantly (75%) single-nucleotide substitutions (18), a predisposition to minor deletions in *GRHPR* is evident.

Conclusions

We analyzed the *GRHPR* genes of four PH2 patients and identified a novel mutation. The c.103delG mutation (in Caucasians), the c.494G>A mutation (in those from the Indian subcontinent), and the c.864_865delTG mutation (particularly in patients of East Asian origin) should be screened when PH2 is suspected.

Supporting Information

The following Supporting information is available for this article:

Figure S1. Nucleotide sequences of intron A and exon 8. Isolated sections of electropherograms are shown. Deduced nucleotide sequences and *GRHPR* codons are shown above each panel. All electropherograms show forward sequence. (a) Control, patient 1, and parents of patient 1; (b) Patient 2 and parents of patient 2; (c) Patients 4 and 5 and their parents. Arrows, affected nucleotide(s); underlining, the first nucleotides of intron H.
Figure S2. Allele discrimination plot of c.864_865delTG (2a) and rs35891798 (2b) is shown. Blue dots indicate homozygous wild type of c.864_865delTG (2a). Green and red dots indicate heterozygous and mutant homozygous case, respectively (2b).

Additional Supporting information may be found in the online version of this article.

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FIELD OF VISION

Internal frontier: The pathophysiology of the small intestine

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Abstract

Even though the small intestine occupies a major portion of the abdominal space and is essential for life, in most pathology textbooks any chapter on small intestinal diseases, especially in human beings, is typically shorter than those for other gastrointestinal organs. Clinical and experimental investigations of the small intestine in various clinical situations, such as nutrition management, obesity interventions, and emergency care, have elucidated several important biological problems associated with the small intestine, the last frontier of gastroenterology. In this issue, a review by Professor Basson and his team at Michigan State University sheds light on the changes in the human small intestine under various conditions based on their clinical and surgical experience. With the advent of recent innovations in enteroscopy, a form of endoscopy used to examine deep within the small intestine, the issue that they highlighted, i.e., mucosal adaptation and atrophy of the human small intestine, has emerged as a major and manageable challenge for gastroenterologists in general, including the readers of the *World Journal of Gastroenterology*.

Key words: Ileum; Jejunum; Small intestine; Atrophy; Adaptation; Enteroscopy

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INVITED COMMENTARY ON HOT ARTICLES

Juan Rosai's Surgical Pathology (9th edition) contains 45 pages on the small intestine, compared with 80 pages on the large intestine[1]. The actual length of the small intestine is much longer than that of the large intestine, and the vital importance of the small intestine is well known. Why then are fewer pages devoted to diseases of the small intestine? Obviously, the access of surgical pathologists and gastroenterologists to the small intestine is limited, compared with access to the large intestine and appendix. Actually, the old version of Morson and Dawson's textbook Gastrointestinal Pathology[2] is fairer: the same number of pages is devoted to the small intestine and to the large intestine, probably because this book is a product of the century during which autopsies made major contributions to our knowledge base. This issue of the *World Journal of Gastroenterology* has a wide-ranging review article on the pathophysiology of the small intestine by Professor Basson and his colleagues[3]. These authors are surgeons, and a tremendous amount of data based on their own clinical experiences and those of others, as well as on animal experiments, are comprehensively discussed in their review. This review article addresses conditions such as starvation and parenteral nutrition in patients with severe trauma or pancreatitis and in patients with postoperative short-gut syndrome for various reasons including bariatric surgery for obesity. Although these

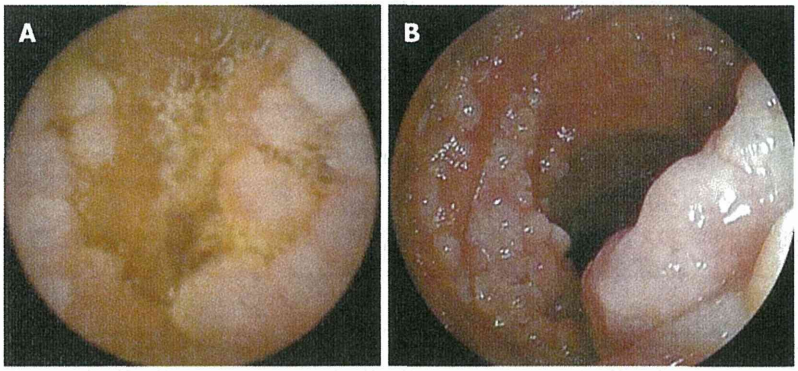

Figure 1 Endoscopy. Images obtained using capsule endoscopy (A) and double-balloon enteroscopy (B) in a 61-year-old man with follicular lymphoma who visited us because of gastrointestinal bleeding from an unknown cause and a hemoglobin concentration of 9.7 g/dL.

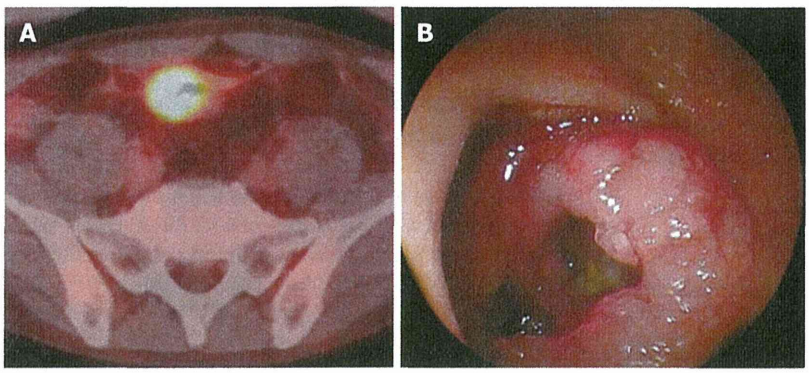

Figure 2 A mass positive by positron emission computed tomography actually was a tumor arising the mucosa of the small intestine of a 48-year-old woman who had suffered Crohn disease. A: Positron emission computed tomography/computed tomography showed an accumulation in site of wall thickening of ileum; B: Image obtained by double-balloon enteroscopy.

conditions are undoubtedly serious, recognition and explicit statements by medical professionals regarding the importance of the small intestine, and of its diseases as a "great burden of human distress", have only recently appeared in medical literature[4]. Most clinical conditions that highlight the importance of the small intestine are familiar only to experts in a particular area of medicine. These conditions are not typically a concern of non-surgical gastroenterologists, such as pathologists who are accustomed to diagnosing neoplasms of the large intestine, grading inflammatory bowel disease, and validating ischemic necrosis of the resected small intestine in cases with an acute abdomen. The small intestine is most often investigated because of its involvement in a disease originating from another organ, such as the mesothelium[5]. Changes in the mucosa of the small intestine itself are an exceptional category among the slides that pile up next to the microscope.

The situation is now changing. As avid readers of *World Journal of Gastroenterology* may have noticed[6-9], recent progress in enteroscopy has enabled areas deep within the small intestine to be reached, providing clinicians active in the wider branches of gastroenterology with the opportunity to evaluate changes in the small intestine, which have previously only been observed by surgeons. This also means that many non-surgeons must commit to the management of the small intestine, based on findings and evaluations associated with specimens of small intestine obtained using these newly developed modalities. For ordinary pathologists, for example, the concepts mainly discussed in this review, i.e., adaptation and atrophy of the small intestine, may be new and unfamiliar. These lesions were encountered only after specific "congenital or acquired disease or medical and surgical intervention" had occurred in the patients. When specimens obtained by enteroscopy become routine in the future, however, the concepts and knowledge of adaptation and atrophy of the small intestine will become an essential "must" for all practitioners including general pathologists.

One of the important conditions that the authors of this review addressed is the change in the small intestine in subjects receiving total parental nutrition. This lifesaving modality has many variations in terms of its nutritional regimen and the effects of these variations on pathophysiology, that is, on the grade of adaptation of the small intestine. These effects on the small intestinal mucosa and the consequent outcomes of individual patients have been thoroughly investigated and published in many scientific articles, but the scientific evidence in human beings remains insufficient according to the