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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

A genetic polymorphism of CYP2C19 is associated with susceptibility to biliary tract cancer

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Abstract

Purpose Cytochrome P450 2C19 (CYP2C19) is clinically important for the metabolism of many therapeutic drugs. CYP2C19 has two main point mutation sites leading to low metabolic capacity. Several CYP enzymes are also important for the metabolism of chemical carcinogens, and several studies have reported associations between CYP polymorphism and cancer susceptibility. Speculating on a potential association between CYP2C19 polymorphism and cancer susceptibility, we conducted this study in two phases. Cell lines of various gastroenterological cancers were screened in the first phase. A clinical investigation was then conducted to confirm the association with the candidate cancer in the second phase.

Methods Genetic polymorphism of CYP2C19 was investigated in a total of 114 cell lines of five gastroenterological cancers. Based on this screening investigation suggesting an association with biliary tract cancer, we conducted a related study by recruiting 65 patients with biliary tract cancer and 566 patients with benign diseases as controls.

Results Among the 114 cell lines investigated, biliary tract cancer was suggested to be most strongly associated with poor metabolizers of CYP2C19. Among 65 patients with biliary tract cancer, 18 (28%) were poor metabolizers of CYP2C19, whereas 87 (15%) of 566 control patients were poor metabolizers. The age- and gender-adjusted odds ratios for intermediate and poor metabolizers regarding the risk of biliary tract cancer were 1.5 (95% CI: 0.8–3.0, $P = 0.17$) and 2.7 (1.3–5.9, $P = 0.006$) compared to extensive metabolizers.

Conclusions A genetic polymorphism of CYP2C19 is associated with susceptibility to biliary tract cancer.

Keywords Biliary tract cancer · CYP2C19 · Genetic polymorphism

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Introduction

Cytochrome P450 2C19 (CYP2C19), one of the principal CYP enzymes, is clinically important for the metabolism of many therapeutic drugs, such as proton-pump inhibitors (PPIs) [1–3], diazepam [4], imipramine [5], propranolol [6], selective serotonin reuptake inhibitors [7], and clopidogrel [8, 9]. CYP2C19 has a genetic polymorphism that influences metabolic capacity and drug effect, and has been a focus of attention for clinical use. It has been reported that CYP2C19 has 19 kinds of polymorphisms; however, most cases can be explained by only two kinds, CYP2C19*2 and CYP2C19*3 [10]. These cause a truncated nonfunctional protein leading to low metabolic capacity [11, 12]. The first is a single base pair (G → A) mutation on exon 5 of CYP2C19 (CYP2C19*2), which creates an aberrant splice site. The second is a single base pair (G → A) mutation on exon 4 of CYP2C19 (CYP2C19*3), which creates

a premature stop codon. The genetically determined CYP2C19 polymorphisms are categorized into three phenotypes by combining these two main mutation sites: extensive metabolizers (EMs), intermediate metabolizers (IMs), and poor metabolizers (PMs).

These phenotypes are strongly related to metabolic capacity. Several reports have documented clinical implications of CYP2C19 phenotypes concerning the metabolism of omeprazole, one of the PPIs. As the area under the plasma concentration–time curve (AUC) is over ten times higher in PMs (5,108.8 ± 319.4) than in EMs (421.2 ± 116.9), intragastric pH is thus much more elevated in PMs (4.47 ± 0.36) than in EMs (2.14 ± 0.10) [13]. The healing rates for both duodenal and gastric ulcers show a CYP2C19 gene dose effect [14, 15]. Furthermore, the cure rates for *Helicobacter pylori* infection, when omeprazole and amoxicillin are used, are 28.6, 60, and 100% in the EMs, IMs, and PMs groups, respectively [15]. A recent report indicated that CYP2C19 polymorphism contributes to variability of platelet response to clopidogrel in patients with myocardial infarction. As CYP2C19 is a key enzyme for converting clopidogrel into an active metabolite, the loss-of-function CYP2C19 is associated with a reduced antiplatelet effect and increased recurrence of ischemic events in patients with clopidogrel therapy [8, 9].

The function of most CYP enzymes is to catalyze the oxidation of organic substances. These substances include chemical carcinogens such as methyleugenol [16] and aromatic amines [17]. CYP enzymes metabolically activate procarcinogens to genotoxic intermediates. Several studies have noted that CYP polymorphism is associated with metabolic activity and cancer susceptibility, and this is more notable in CYP1A1, CYP2D6, and CYP2E1. Most CYP enzymes can metabolize multiple substrates, and they can catalyze multiple reactions, which accounts for their central importance in metabolizing an extremely large number of endogenous and exogenous molecules. Therefore, CYP2C19 is also suspected to play an important role in either detoxication or inactivation of potential carcinogens, or the bioactivation of some environmental procarcinogens to toxic DNA-binding metabolites. Consequently, CYP2C19 polymorphism associated with metabolic activity could cause a susceptibility to several forms of cancer. Indeed, there have been several reports concerning CYP2C19 polymorphism and cancer susceptibility, such as esophageal, stomach, and lung cancers [18] and leukemia [19]. However, the mechanism and the target carcinogen have not been elucidated, and there have been no reports concerning CYP2C19 polymorphism and other forms of cancer.

Speculating on a potential association between CYP2C19 polymorphism and susceptibility to other forms of cancer, we planned this study in two phases. Cell lines of various gastroenterological cancers were screened in the first phase. They were genotyped for CYP2C19, and the candidate cancer was determined according to the proportions of CYP2C19 genotypes. In the second phase, a clinical investigation was conducted to confirm an association with the candidate cancer. The patients with the candidate cancer were genotyped and compared to control patients.

Methods

Screening study by cell lines

Cell lines

The following cell lines were genotyped for CYP2C19 in the first step of this study (gastric cancer 33 cell lines, colorectal cancer 32, hepatic cancer 15, pancreatic cancer 25, and biliary tract cancer 10):

Gastric cancer AGS, AZ-521, AZ-H6C, ECC10, ECC12, FU97, GCIY, H-111-TC, HGC-27, Hs746T, HuG1-P1, HuG1-N, IM95, KATOIII, KE39, KE97, MKN1, MKN7, MKN45, MKN74, NCI-N87, NUGC-2, NUGC-3, NUGC-4, SCH, SH-10-TC, SH101-P4, SNU-1, SNU-5, SNU-16, Takigawa, TGBC11T, TMK-1.

Colorectal cancer Caco-2, CCK-81, COLO201, COLO205, COLO320, DLD-1, HCT15, HCT116, LoVo, LS123, LS180, LS174T, OUMS-23, PMF-ko14, RKO, RKO-AS45-1, RKO-E6, SK-CO-1, SW403, SW480, SW1116, SW620, SW837, SW1417, T84, WiDr, SNU-C1, HCI H630, CaR-1, HT29, RCM-1, SW48.

Hepatic cancer Alexander, Hep3B, HepG2, HLE, HLF, HT17, HuH1, HuH6, HuH7, JHH-1, JHH-4, JHH-7, RBE, SK-HEP-1, SNU-398, SNU-449, SSP-25.

Pancreatic cancer AsPC-1, BxPC-3, Capan-1, Capan-2, CFPAC-1, HPAF-II, Hs700T, Hs766T, KLM-1, KP1N, KP2N, KP3N, KP-4, MIA paca, NOR-P1, Panc 02.03, Panc 03.27, Panc 05.04, Panc 08.13, Panc 10.05, PANC-1, PL45, SU.86.86, sw1990, T3M-4.

Biliary tract cancer HuCCT1, MEC, NOZ, OZ, RBE, SSP-25, TFK-1, TGBC24T, TKKK, TGBC1TK.

CYP2C19 genotyping

DNA was extracted from each cell line using a commercially available kit (QIAamp® DNA Mini Kit; Qiagen, Valencia, CA, USA). A polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method was applied as described by de Morais et al. [11, 12] with a minor modification [10]. In brief, the two regions including the point mutation sites were amplified by PCR methods as

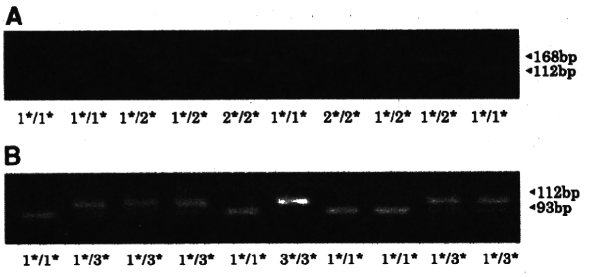

Fig. 1 Samples for CYP2C19 polymorphism analysis. **a** Samples for CYP2C19*2 analysis. PCR products were digested with *MspI*; the 168-bp PCR products of the WT were cut into 112- and 51-bp fragments, but not in CYP2C19*2. **b** Samples for CYP2C19*3 analysis. PCR products were digested with *Bam*HI; the 119-bp PCR products of the WT were cut into 93- and 26-bp fragments, but not in CYP2C19*3

described below. The amplification was performed using a thermocycler for 40 cycles consisting of denaturation at 95°C for 30 s, annealing at 53°C for 30 s, and extension at 72°C for 30 s. An initial denaturation step at 95°C for 10 min and a final extension step at 72°C for 5 min were also performed. The forward primer 5′-AATTACAACCAGA GCTTGGC-3′ and the reverse primer 5′-TATCACTTTCC ATAAAAGCAAG-3′ were used to detect the CYP2C19*2 allele, and the PCR products were digested with Msp I; the 169-bp PCR products of the wild type (WT) were cut into 117- and 51-bp fragments, but not in CYP2C19*2. The forward primer 5′-AACATCAGGATTGTAAGCAC-3′ and the reverse primer 5′-TCAGGGCTTGGTCAATATAG-3′ were used to detect the CYP2C19*3 allele, and the PCR products were digested with *Bam*HI; the 119-bp PCR products of the WT were cut into 93 and 26-bp fragments, but not in CYP2C19*3. The digested PCR products were analyzed on 3% agarose gels and stained with ethidium bromide (Fig. 1).

Cases that were homozygous for either the CYP2C19*2 or CYP2C19*3 mutation (*2/*2 or *3/*3) and heterozygous for CYP2C19*2 and CYP2C19*3 (*2/*3) were categorized as PMs. Cases that were heterozygous for the WT and mutation (*1/*2 or *1/*3) were categorized as IMs, and those homozygous for the WT (*1/*1) were categorized as EMs.

Clinical study in patients with biliary tract cancer

Participants

Sixty-five patients with biliary tract cancer diagnosed at the University of Tokyo Hospital between March 2002 and December 2007 were included in this study. The diagnosis of biliary tract cancer was confirmed based on radiological and pathological studies. On the other hand, 566 Japanese patients without cancer who consulted the University of Tokyo Hospital within the same period were entered as a control group. The genomic DNA of these patients was made available after obtaining written informed consent for genotyping according to the Declaration of Helsinki. The protocol was approved by the local ethics committee.

CYP2C19 genotyping

DNA was extracted from each patient's whole blood using a commercially available kit (Puregene DNA Isolation Kit; Gentra Systems, Minneapolis, MN, USA). Genotyping of CYP2C19 was conducted by PCR-RFLP as described for the cell lines.

Data analysis

The Hardy–Weinberg equilibrium of allele frequencies at individual loci was assessed by comparing the observed and expected genotype frequencies using the chi-square test. Clinical parameters were evaluated using the two-tailed *t* test and the chi-square test. Differences in distributions of CYP2C19 genotypes and phenotypes between the controls and the biliary tract cancer cases were evaluated using the chi-square test. The effects of the CYP2C19 genotype on the risk of developing biliary tract cancer were expressed as odds ratios (ORs) adjusted in multivariate logistic regression models that included age and gender. For all tests, a P value of less than 0.05 was considered statistically significant. All data analyses were conducted using SAS software (version 9; SAS Institute, Inc., Cary, NC, USA).

Results

Screening study by cell lines

Among the cell lines of the five cancers, the frequency of PMs for CYP2C19 was 27.3% (9/33) in gastric cancer, 0% (0/32) in colorectal cancer, 6.7% (1/15) in hepatic cancer, 12.0% (3/25) in pancreatic cancer, and 60.0% (6/10) in biliary tract cancer (Table 1). The frequency of PMs in gastric cancer was comparable to that stated in a previous report suggesting an association between PMs of CYP2C19 and gastric cancer [20]. Additionally, the prevalence of PMs among biliary tract cancer cell lines seemed extremely high, even when compared to the gastric cancer cell lines. Based on this screening study by cell lines, biliary tract cancer was raised as a candidate for clinical study to confirm the association with CYP2C19 polymorphism.

Clinical study in patients with biliary tract cancer

The characteristics of the biliary tract cancer cases and the controls are shown in Table 2. Mean age was higher in the

Table 1 Frequencies of CYP2C19 phenotypes in various cancer cell lines

CYP2C19 phenotype	Gastric cancer ($n = 33$)	Colorectal cancer ($n = 32$)	Hepatic cancer ($n = 15$)	Pancreatic cancer ($n = 25$)	Biliary tract cancer ($n = 10$)
EMs	13 (39.4%)	23 (71.9%)	8 (53.3%)	14 (56.0%)	2 (20.0%)
IMs	11 (33.3%)	9 (28.1%)	6 (40.0%)	8 (32.0%)	2 (20.0%)
PMs	9 (27.3%)	0 (0%)	1 (6.7%)	3 (12.0%)	6 (60.0%)

Gastric cancer: AGS, AZ-521, AZ-H6C, ECC10, ECC12, FU97, GCIY, H-111-TC, HGC-27, Hs746T, HuG1-P1, HuG1-N, IM95, KATOIII, KE39, KE97, MKN1, MKN7, MKN45, MKN74, NCI-N87, NUGC-2, NUGC-3, NUGC-4, SCH, SH-10-TC, SH101-P4, SNU-1, SNU-5, SNU-16, Takigawa, TGBC11T, TMK-1

Colorectal cancer: Caco-2, CCK-81, COLO201, COLO205, COLO320, DLD-1, HCT15, HCT116, LoVo, LS123, LS180, LS174T, OUMS-23, PMF-ko14, RKO, RKO-AS45-1, RKO-E6, SK-CO-1, SW403, SW480, SW1116, SW620, SW837, SW1417, T84, WiDr, SNU-C1, HCI H630, CaR-1, HT29, RCM-1, SW48

Hepatic cancer: Alexander, Hep3B, HepG2, HLE, HLF, HT17, HuH1, HuH6, HuH7, JHH-1, JHH-4, JHH-7, RBE, SK-HEP-1, SNU-398, SNU-449, SSP-25

Pancreatic cancer: AsPC-1, BxPC-3, Capan-1, Capan-2, CFPAC-1, HPAF-II, Hs700T, Hs766T, KLM-1, KP1N, KP2N, KP3N, KP-4, MIA paca, NOR-P1, Panc 02.03, Panc 03.27, Panc 05.04, Panc 08.13, Panc 10.05, PANC-1, PL45, SU.86.86, sw1990, T3 M-4

Biliary tract cancer: HuCCT1, MEC, NOZ, OZ, RBE, SSP-25, TFK-1, TGBC24T, TKKK, TGBC1TK

EMs, extensive metabolizers, consisting of *1/*1; *IMs*, intermediate metabolizers, consisting of *1/*2 and *1/*3, and *PMs*, poor metabolizers, consisting of *2/*3, *2/*2, and *3/*3

Table 2 Baseline characteristics of the biliary tract cancer cases and the controls

	Biliary tract cancer ($n = 65$)	Controls ($n = 566$)	*P* value
Mean age ± SD	66.4 ± 10.8	56.9 ± 12.8	0.0001†
Sex			
Men/women	44/21	317/249	0.07‡
Stage			
I or II/III or IV	15/50		
Differentiation[a]			
Well or moderately/poorly	41/8		
Primary lesion			
Intrahepatic/perihilar/distal/Vater	13/23/8/6		
Gallbladder	15		
Risk factor (primary sclerosing cholangitis)			
+/−	0/65		

SD standard deviation

† *P* values were calculated using the Student *t* test

‡ *P* values were calculated using the chi-square test

[a] Diagnosis of biliary tract cancer was confirmed in 49 out of 65 participants based on the pathological studies

cases with biliary tract cancer than in controls. The primary origins of biliary tract cancer were in the intrahepatic bile duct (13), perihilar bile duct (23), distal extrahepatic bile duct (8), ampulla of Vater (6), and gallbladder (15).

Distributions of CYP2C19 genotypes and phenotypes among the biliary tract cancer cases and the controls are illustrated in Tables 3 and 4. The controls (317 men and 249 women; mean age, 56.9 years, SD = 12.8; age range, 38–84) consisted of 36.2% EMs, 48.2% IMs, and 15.5% PMs. The distribution of phenotypes in the controls was almost the same as the results for healthy Japanese patients in the previous report [10]. The distribution of alleles in the controls was consistent with Hardy–Weinberg equilibrium (Table 4); therefore, the controls in this study were assumed to provide reasonable validity for the aim of the current study. On the other hand, 65 participants (44 men and 21 women; mean age, 66.7 years, SD = 10.6; age range, 41–85) with biliary tract cancer were investigated for CYP2C19 genotyping. Of these 65 participants, 16 (24.6%) were EMs, 31 (47.7%) were IMs, and 16 (27.7%) were PMs (Table 3). There was a significant difference in the distribution of CYP2C19 phenotypes between the biliary tract cancer cases and the controls ($P = 0.025$). The adjusted OR (95% CI) of PMs to EMs was 2.7 (1.3–5.9) (Table 3). The IMs had an OR of 1.5 (0.8–3.0) to EMs, suggesting an increasing trend for the risk of biliary tract cancer as the metabolic capacity of CYP2C19 decreased. There is no significant difference in patient characteristics, including age at diagnosis, sex, tumor stage, and tumor differentiation, between the three CYP2C19 phenotypes (Table 5). Among PMs, genotype *2/*3 showed a higher OR of 4.7 (2.0–11) than genotype *2/*2 (OR = 1.3, 0.3–3.9) (Table 4). Both the CYP2C19*2 and *3 alleles showed an increased risk for biliary tract cancer compared to allele *1, with ORs (95% CI) of 1.6 (1.0–2.4; $P = 0.03$) and 1.7 (1.0–2.90; $P = 0.04$), respectively (Table 6). Frequencies of CYP2C19 phenotypes according to the location of biliary tract cancer are shown in Table 7. When classifying "biliary tract cancer" into "bile duct cancer" and "gallbladder cancer," the OR for PMs compared with EMs and IMs combined was calculated to be 2.72 (0.91–8.14) in the

Table 3 Distribution of CYP2C19 phenotypes among the biliary tract cancer cases and the controls

[a] Odds ratio (OR) adjusted in multivariate logistic regression models including age and gender

CYP2C19 phenotype	Biliary tract cancer (n = 65)	Controls (n = 566)	OR[a]	95% CI	P value
EMs	16 (24.6%)	205 (36.2%)			
IMs	31 (47.7%)	273 (48.2%)	1.51	0.79–2.96	0.173
PMs	18 (27.7%)	88 (15.5%)	2.74	1.29–5.87	0.0058

Table 4 Distribution of CYP2C19 genotypes among the biliary tract cancer cases and the controls

[a] Odds ratio (OR) adjusted in multivariate logistic regression models including age and gender

CYP2C19 genotype	Biliary tract cancer (n = 65)	Controls (n = 566)	OR[a]	95% CI	P value
*1/*1	16 (24.6%)	205 (36.2%)			
*1/*2	23 (35.4%)	193 (34.1%)	1.59	0.80–3.22	0.19
*1/*3	8 (12.3%)	80 (14.1%)	1.37	0.52–3.38	0.51
*2/*2	4 (6.2%)	40 (7.1%)	1.29	0.35–3.89	0.68
*2/*3	14 (21.5%)	38 (6.7%)	4.73	2.02–11.07	0.001
*3/*3	0 (0%)	10 (1.8%)	–	–	–

Table 5 Patient characteristics among the CYP2C19 phenotypes

	EMs	IMs	PMs	P value
Mean age ± SD	69.4 ± 11.0	64.1 ± 10.5	67.8 ± 11.1	0.23[†]
Sex				0.45[‡]
Men/women	9/7	23/8	12/6	
Stage				0.74[‡]
I or II/III or IV	4/12	8/23	3/15	
Differentiation[a]				0.42[‡]
Well or moderately/ poorly	8/3	19/4	14/1	

SD standard deviation

[†] P values were calculated using the Student t test

[‡] P values were calculated using the chi-square test

[a] The diagnosis of biliary tract cancer was confirmed based on pathological studies in 49 out of 65 participants

gallbladder cancer group. This was a notable trend in contrast to the bile duct cancer group (OR = 1.91, 0.98–3.74) and the whole biliary tract cancer group (OR = 2.08, 1.15–3.75).

Discussion

Biliary tract cancer is a highly fatal disease with poor prognosis. It is a relatively rare form of cancer in Western countries. However, it is more common in Japan than in Western countries, and approximately 16,000 people die in Japan from this disease each year. There is no potentially curative treatment except surgery. Most patients have advanced and inoperable disease at the time of diagnosis. Chronic inflammation is considered a risk factor for biliary tract cancer, including primary sclerosing cholangitis and congenital liver malformation. Previously reported genetic risk factors are p53 mutation [21] and HLA-DR4 [22] in gallbladder cancer, albeit with insufficient epidemiologic evidence. In this study, CYP2C19 PMs genotypic status showed a higher risk for biliary tract cancer, suggesting that CYP2C19 is involved in the detoxification of some carcinogens for biliary tract cancer. Various chemicals metabolized in the liver flow into the intestine thorough the bile duct. Thus, the bile duct can be strongly affected by bile carcinogens. If such carcinogens are inactivated or metabolized by CYP2C19, their concentrations in the bile should be higher in PMs of CYP2C19 than in EMs. It is important to acknowledge that gallbladder cancer may have a stronger association with CYP2C19 polymorphism. The OR for PMs compared with EMs and IMs combined was calculated to be 2.72 (0.91–8.14) in the gallbladder cancer group. This was a notable trend in contrast to the bile duct cancer group (OR = 1.91, 0.98–3.74) and the whole biliary tract cancer group (OR = 2.08, 1.15–3.75), which might be due to carcinogens concentrated by the gallbladder. On the other hand, it has been reported that gallstone may increase the risk of developing gallbladder cancer [23]. We investigated CYP2C19 polymorphism in cases with gallstones. Of the 30 participants with gallstones, 12 (40.0%) were EMs, 13 (43.3%) were IMs, and 5 (16.7%) were PMs. There was no significant difference in the distribution of CYP2C19 phenotypes compared with the controls (P = 0.87). This result suggests that there is an association between CYP2C19 polymorphism and cancer risk, but not for gallstone.

There is a regional difference in the incidence of biliary tract cancer. The highest incidence is observed among people in Eastern Asia, such as Japan and Korea, Chile, and

Table 6 Influences of CYP2C19 alleles on the biliary tract cancer cases

[a] Odds ratio (OR) adjusted in multivariate logistic regression models including age and gender

CYP2C19 allele	Biliary tract cancer (n = 65)	Control (n = 566)	OR[a]	95% CI	P value
*1	63 (48.5%)	683 (60.3%)			
*2	45 (34.6%)	311 (27.5%)	1.57	1.05–2.35	0.03
*3	22 (16.9%)	138 (12.2%)	1.73	1.03–2.90	0.04

Table 7 Frequencies of CYP2C19 phenotypes according to the location of biliary tract cancer

	EMs	IMs	EMs + IMs	PMs	OR (95% CI)[a]	P[†]
Control (n = 566)	205 (36.2%)	273 (48.2%)	478 (84.5%)	88 (15.5%)	1.0	
Biliary tract cancer (n = 65)	16 (24.6%)	31 (47.7%)	47 (72.3%)	18 (27.7%)	2.08 (1.15–3.75)	0.02
Bile duct cancer	11	26	37	13	1.91 (0.98–3.74)	0.05
Intrahepatic	4	6	10	3		
Perihilar	3	16	19	4		
Distal	3	2	5	3		
Vater	1	2	3	3		
Gallbladder	5	5	10	5	2.72 (0.91–8.14)	0.17

[†] P values were calculated using the chi-square test

[a] Odds ratios (ORs) were calculated between the PMs and the EMs and IMs combined based on the control group

Native Americans. A low incidence is reported in the Western world, such as in the US and the UK. The annual mortality rate per 100,000 is approximately 13.0 in Japan; in contrast, it is 1.2 in the US and 1.0 in the UK [24]. Japan has a markedly higher incidence of biliary tract cancer than that of the Western world. It has been reported that this wide geographical variation may be related to a much longer history of gallstone disease and different dietary patterns [25]. Meanwhile, concerning genetic factors, it has been reported that p53 mutation and microsatellite instability differ in patients with biliary tract cancer among high-incidence areas, so geographic variation may exist in the process of biliary tract carcinogenesis [21]. On the other hand, there are interracial differences in CYP2C19 genotype profiles. Frequencies of EMs, IMs, and PMs in Asians are approximately 30–40%, 40–50%, and 14–22%, whereas those in Caucasians are approximately 70–75%, 20–25%, and 2–5%, respectively [10, 26, 27]. The higher PMs frequency of the CYP2C19 genotype in the Japanese population may cause a higher incidence of biliary tract cancer compared to Caucasians if CYP2C19 PMs status is a risk factor for developing biliary tract cancer.

The incidence of biliary tract cancer also varies within a country. In Japan, for example, the incidence of biliary tract cancer is higher in eastern than in western Japan. Niigata prefecture has the highest incidence in Japan [28], and there are also regional differences within prefectures [29]. Mortality is high on the Niigata plain and low in other parts of Niigata. Based on epidemiological studies, the presence of environmental water pollution by agricultural chemicals is likely to be associated with the occurrence of biliary tract cancer [29]. Therefore, an association between these chemicals and CYP2C19 enzyme should be examined in a future study.

Most CYP enzymes can metabolize multiple substrates, and certain subtypes of CYP may be involved in the metabolism of carcinogens. Polymorphisms of other types of CYP are known to be associated with the development of various cancers, including lung cancer [30–33], breast cancer [34], esophageal cancer [35, 36], ovarian cancer [34], and prostate cancer [37]. CYP1A1 and CYP2E1 polymorphisms are frequently reported to be related to chemical carcinogenesis and susceptibility to other forms of cancer, and the Japanese are known to have a higher incidence of such polymorphisms. We examined these polymorphisms. Concerning CYP1A1, we examined CYP1A1*3 in codon 462 of exon 7, which results in an amino acid substitution of Ile for Val in the catalytic region of the CYP1A1 protein, by the previously reported method [38]. Concerning CYP1A1*3 polymorphism, a previous study has reported that 233 (65.1%) of 388 healthy Japanese were A/A, 108 (30.2%) were A/G, and 17 (4.7%) were G/G . We were able to genotype 59 of 65 participants with biliary tract cancer: 36 (61.0%) were A/A, 21 (35.6%) were A/G, and 2 (3.4%) were G/G. Furthermore, in regards to CYP2E1, we examined the CYP2E1*5 polymorphism ascribed to the RsaI site in the 5′-flanking region by the PCR-RFLP method described by Le Marchand et al. [39]. Concerning CYP2E1*5

polymorphism, it has been reported that 455 (58.5%) of 778 healthy Japanese were c1/c1, 279 (35.9%) were c1/c2, and 44 (5.7%) were c2/c2. We were able to genotype 61 of 65 participants with biliary tract cancer: 33 (54.1%) were c1/c1, 27 (44.3%) were c1/c2, and 1 (1.6%) were c2/c2. There was no significant difference in the distributions of polymorphism in both CYP1A1 and CYP2E1 between the biliary tract cancer cases and the controls. This result may suggest that CYP2C19 polymorphism is an independent risk factor for biliary tract cancer, and that this is unique to this type of cancer.

Cancer cells include many somatic mutations. Therefore, there is a possibility of incorrect estimation during CYP2C19 genotyping if somatic mutations exist at the SNP site. We also confirmed that the CYP2C19 genotypes were identical between samples extracted from cancer lesions and noncancer lesions in a subpopulation of the patients. These results were equivalent to those obtained from an analysis of patient whole blood (data not shown). Somatic mutations at the SNP site of CYP2C19 did not seem to be frequent in cancers. Thus, the CYP2C19 genotyping test is believed to be useful for screening individuals at a higher risk for biliary tract cancer.

In conclusion, we have demonstrated that a genetic polymorphism of CYP2C19 is associated with susceptibility to biliary tract cancer. However, our results must be interpreted within the context of the limitations of the study. First of all, the control group of this study did not comprise healthy individuals, but patients with benign diseases. We could not evaluate the effects of CYP2C19 genotype status on biliary tract cancer development independently. Second, the sample size was rather small, reflecting the low incidence of biliary tract cancer. When classifying biliary tract cancer according to the primary lesion, there is a possibility that there are different mechanisms of cancer development. Our findings must be confirmed by future studies with a larger number of patients with biliary tract cancer and healthy controls, and verified in a prospective study.

Conflict of interest statement There was no conflict of interest in this study.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Bezafibrate for the treatment of primary sclerosing cholangitis

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Abstract

Background It is known that bezafibrate decreases serum alkaline phosphatase (ALP) in patients with hyperlipidemia, and the efficacy of this drug for the treatment of primary biliary cirrhosis has been confirmed. However, there has been little evidence of its efficacy for the treatment of primary sclerosing cholangitis (PSC).

Methods Bezafibrate (400 mg/day) was orally administered to 7 consecutive patients with PSC, and we analyzed their clinical features and the drug efficacy in terms of the effect on hepatobiliary enzymes, including ALP, gamma-glutamyl transpeptidase (γ-GTP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) after 6 months. The latest hepatobiliary enzyme levels were also evaluated.

Results In 3 patients (effective group), the levels of all hepatobiliary enzymes had decreased after 6 months. Mean ALP had decreased to approximately 40% of the baseline in this group. The efficacy of bezafibrate was observed for a long period (range, 8–27 months) in these 3 patients. There seemed to be no definite association between the efficacy of bezafibrate and the clinical features in the short term.

Conclusions This study showed that bezafibrate could lower the levels of hepatobiliary enzymes in about half of a cohort of patients with PSC.

Keywords Primary sclerosing cholangitis · Drug therapy · Bezafibrate

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Introduction

Primary sclerosing cholangitis (PSC) is a chronic inflammatory cholestatic liver disease of unknown etiology, characterized by chronic inflammation and obliterative fibrosis of the intra- and extra-hepatic biliary tree [1]. The disease is slowly progressive, even in asymptomatic patients, usually leading to biliary cirrhosis and its complications over a 10–15-year period [2]. Although ursodeoxycholic acid (UDCA) is widely used for PSC patients and UDCA can lead to improvements in serum hepatobiliary enzymes [3], a randomized controlled trial in which 198 patients were enrolled demonstrated no effect of UDCA on symptoms, quality of life, or transplant-free survival [4]. Corticosteroids are also used in some PSC patients, but corticosteroids do not seem to be effective in typical PSC and have considerable risks in this population (osteoporosis, increased susceptibility to infection) [3]. Currently, there is no medical treatment for PSC with enough evidence to show a delay in disease progression, and new therapy is needed.

Bezafibrate is a commonly used medication for hyperlipidemia, and reduction in serum alkaline phosphatase (ALP) was initially shown as a well-documented side effect of fibric acid derivatives [5]. It was reported that bezafibrate decreased serum hepatobiliary enzymes in hyperlipidemic patients without any liver diseases [6]. Subsequently, bezafibrate was first used for the treatment of primary biliary

cirrhosis (PBC) in 1999, and it was demonstrated that this drug had the beneficial effect of lowering hepatobiliary enzyme levels [7]. Although PBC and PSC are different disease entities, cholestasis finally causes cirrhosis in both diseases. Bezafibrate was later used for the treatment of PSC, and a beneficial effect was reported in 2002 [8]. Until now, only 7 cases of PSC treated with bezafibrate have been reported in the English-language literature, from 2 groups [9, 10]. These studies reported limited numbers of cases with a beneficial effect, and information about patient characteristics was not clarified. In the present study, we report our experience of 7 consecutive patients with PSC treated with bezafibrate (including cases in which the treatment was ineffective); we also examined factors associated with the efficacy of bezafibrate.

Methods

In this study the findings in seven patients with PSC who were treated with bezafibrate and followed at the University of Tokyo Hospital or the Japanese Red Cross Medical Center between November 2006 and June 2008 were analyzed retrospectively. We used diagnostic criteria published from the Mayo Clinic in 2003 [11], but excluded patients with sclerosing cholangitis with autoimmune pancreatitis (AIP) who fulfilled the diagnostic criteria of AIP proposed by the Mayo Clinic [12] or the revised criteria of the Japan Pancreas Society [13]. Clinical features and laboratory data were reviewed from medical records. Other medications used for PSC during the period of administration of bezafibrate were also reviewed.

Bezafibrate was administered orally twice a day, for a total dose of 400 mg/day. Changes in the levels of ALP, gamma-glutamyl transpeptidase (γ-GTP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were evaluated. In order to assess the efficacy of bezafibrate for reducing the serum enzyme levels, a reduction index (RI) was calculated: RI = postadministration serum level/preadministration serum level. The primary endpoint of the study was the RI for each hepatobiliary enzyme after 6 months of bezafibrate administration. The secondary endpoint was the latest RI for ALP in the observation period as of February 2009.

Informed consent was obtained orally from each patient before administering bezafibrate.

Results

All patients were followed for at least 6 months after the introduction of bezafibrate. The clinical features of the patients at the time of the introduction of bezafibrate are summarized in Table 1. There were 3 men and 4 women.

Table 1 Baseline characteristics

	Age/sex	Age of at onset (years)	Duration of disease (months)	Other medications				Plt (10^9/L)	PT-INR	Alb (g/dL)	ALP (IU/L)	γ-GTP (IU/L)	AST (IU/L)	ALT (IU/L)	T. bil (mg/dL)	T. chol (mg/dL)	TG (mg/dL)	Child-Pugh class
				UDCA (mg)	PSL (mg)	UC	CCA											
Case 1	34/F	33	16	600	–	+	–	281	1.25	4.2	877	481	83	167	0.9	166	71	A
Case 2	80/F	80	6	–	–	–	–	218	1.14	3.6	315	179	38	27	0.5	177	98	A
Case 3	73/M	69	48	600	–	–	+	258	1.05	3.3	507	155	58	41	2	201	158	B
Case 4	58/M	57	18	900	–	–	–	324	1.05	2.7	1072	277	70	62	2.8	294	132	B
Case 5	31/M	30	19	600	–	–	–	270	0.94	4.8	295	214	30	49	0.6	–	–	A
Case 6	35/F	32	39	600	–	–	–	286	0.91	4.3	438	126	27	42	0.7	212	58	A
Case 7	69/F	61	97	900	6	–	–	310	0.89	3.4	1077	227	48	67	2.2	191	–	B

UDCA ursodeoxycholic acid, *PSL* prednisolone, *UC* ulcerative colitis, *CCA* cholangiocarcinoma, *Plt* platelets, *PT* prothrombin time
Alb albumin, *ALP* alkaline phosphatase, *γ-GTP* Gamma-glutamyl transpeptidase, *AST* aspartate aminotransferase
ALT alanine aminotransferase, *T. bil* total bilirubin, *T. chol* total cholesterol, *TG* triglyceride

The mean age at onset was 52 years (range, 30–80 years). The mean duration of the disease was 35 months (range, 6–97 months). In Case 6, bezafibrate was stopped after 3 months because of the rapid elevation of hepatobiliary enzymes, and UDCA was then administered. In Case 1, UDCA was added after 4 months of bezafibrate administration. In Cases 3–5, and 7, bezafibrate was added to UDCA treatment. Overall, UDCA was used in 6 patients.

Changes in hepatobiliary enzyme levels during the administration of bezafibrate are shown in Table 2, and these changes, expressed as the RIs, are shown in Fig. 1. In the patients overall, the mean RIs for ALP, γ-GTP, AST, and ALT after 6 months were 0.69, 0.83, 1.35, and 0.95, respectively. In Cases 1–3, the RIs for all hepatobiliary enzymes were below 1.0 after 6 months of bezafibrate administration; therefore, we considered bezafibrate was effective in these patients. The mean RIs for ALP, γ-GTP, AST, and ALT after 6 months were 0.41, 0.38, 0.49, and 0.30, respectively, in this effective group; also, improvements in each hepatobiliary enzyme level had been achieved after 3 months of bezafibrate administration in this group.

Table 2 Changes in hepatobiliary enzymes enzyme levels in each patient

	Time (months)		
	0	3	6
ALP (IU/L)			
Case 1	877	275	134
Case 2	315	126	132
Case 3	507	294	336
Case 4	1072	1040	806
Case 5	295	190	215
Case 6	438	594	446
Case 7	1077	972	1204
γ-GTP (IU/L)			
Case 1	481	248	66
Case 2	179	28	26
Case 3	155	130	131
Case 4	277	654	532
Case 5	214	81	189
Case 6	126	206	109
Case 7	227	218	227
AST (IU/L)			
Case 1	83	43	34
Case 2	38	20	21
Case 3	58	29	30
Case 4	70	251	268
Case 5	30	25	47
Case 6	27	39	22
Case 7	48	72	86
ALT (IU/L)			
Case 1	167	41	29
Case 2	27	11	9
Case 3	41	16	16
Case 4	62	181	156
Case 5	49	38	59
Case 6	42	63	36
Case 7	67	78	78

The latest RIs for ALP are shown in Table 3. The maximum observation period was 27 months. In the effective group, the RIs were below 1.0 even after a long period of bezafibrate administration. In Cases 4, 5, and 7, whose ALP decreased slightly after 3 or/and 6 months, bezafibrate was continued thereafter; however, the ALP increased again in all 3 patients. In Case 6, UDCA was administered for 8 months after bezafibrate discontinuation, and the latest RIs for ALP, γ-GTP, AST, and ALP were 0.97, 0.80, 0.78, and 0.69, respectively.

Bezafibrate was administered as the first medication in 3 patients (Cases 1, 2, and 6), and the effect of bezafibrate as a single-agent therapy was assessable in these patients. The RIs for ALP in each of these patients after 3 months of bezafibrate monotherapy were 0.31, 0.40, and 1.36 (mean, 0.69). In Case 2, bezafibrate monotherapy was continued for 17 months, and the latest RI for ALP was 0.49.

As for liver function, the Child-Pugh class was A in 4 patients and B in 3 (Table 1). Bezafibrate was effective in 2 of the 4 patients with Child A class liver function, and in 1 of the 3 patients with Child B class liver function. There seemed to be no association between the efficacy of bezafibrate and the Child-Pugh class. There were no remarkable changes in platelet counts, prothrombin time (PT)-INR, albumin, or total bilirubin during the treatment with bezafibrate (data not shown).

Discussion

In the present study, bezafibrate was administered to 7 consecutive patients with PSC, and improvements in the levels of all hepatobiliary enzymes were observed in 3 patients (43%). Bezafibrate was not effective in all the patients, although only effective cases were described in the previous reports [9, 10]. In the effective group in the present study, the effect of bezafibrate was observed rapidly, in 1 month, and lasted for a long period (Table 3). Meanwhile, in the ineffective group, except for Case 6, the RIs for ALP did not fall below 1.0 after 9–26 months of administration. The mid- to long-term effect of bezafibrate may be predicted from laboratory data after 6 months' administration. The levels of hepatobiliary enzymes were normalized after 6 months in 2 patients in our effective group. Porayko et al. showed in their study that

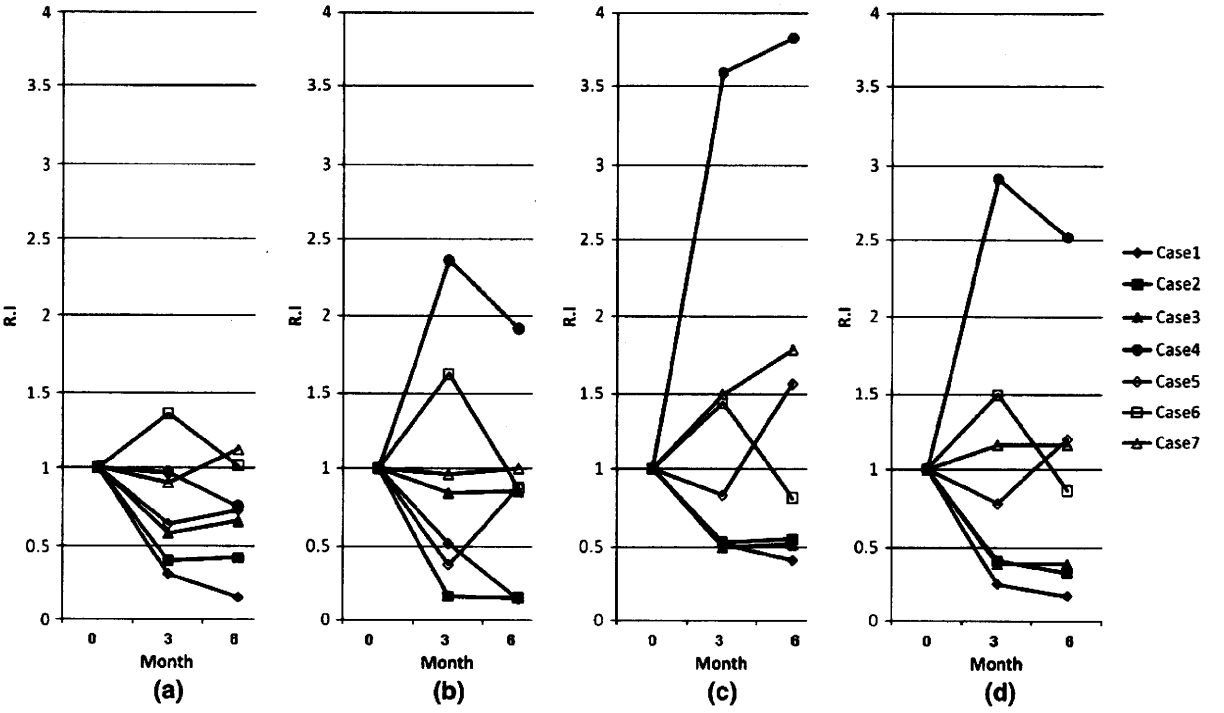

Fig. 1 Changes in hepatobiliary enzyme levels in each patient. Reduction indexes for **a** alkaline phosphatase, **b** gamma-glutamyl transpeptidase, **c** aspartate aminotransferase, and **d** alanine aminotransferase

Table 3 The latest reduction index values of alkaline phosphatase

	Observation period (months)	RI
Effective group		
Case 1	27	0.15
Case 2	17	0.49
Case 3	8	0.55
Ineffective group		
Case 4	26	1.22
Case 5	18	1.26
Case 6	11	0.97
Case 7	9	1.24

RI reduction index

asymptomatic PSC patients with comparatively high levels of AST or ALP had short survival times [2]. Thus, bezafibrate, which can lower the levels of AST and ALP in some patients with PSC, may delay disease progression.

Assessment of the effect of bezafibrate as single-agent therapy was possible in 3 patients in our study. The mean RI for ALP after 3 months of bezafibrate monotherapy in these 3 patients was 0.69. The RIs for ALP with UDCA monotherapy for PSC at standard doses were reported to be 0.53–0.75 in previous trials [14–16]. Bezafibrate monotherapy may have a beneficial effect similar to that of UDCA.

As for combination therapy of bezafibrate and UDCA, 3 of 4 patients in the present study for whom bezafibrate was added to UDCA therapy did not show a definite beneficial effect, although 2 of 3 patients in whom bezafibrate was administered prior to UDCA therapy showed a beneficial effect. This may mean that the use of bezafibrate for patients who are refractory to UDCA is not effective. However, it should be considered that bezafibrate plus UDCA therapy was administered in patients with Child B class liver function in the present study. From the viewpoint of drug therapy for PSC, UDCA eventually decreased the level of all hepatobiliary enzymes in Case 6, a patient with Child A class liver function. Overall, drug therapy was effective in 3 of our 4 patients with Child A class liver function, and in 1 of 3 with Child B class liver function. In patients without cirrhosis, it was reported that bezafibrate had an additional effect in UDCA-resistant PBC patients [17]. Similarly, oral vancomycin was reported to be effective in children with noncirrhotic PSC [18]. Thus, it is possible that drug therapy for both PSC and PBC may be more effective in patients with better liver function.

It has been reported that Japanese patients with PSC can be categorized into 2 groups [19, 20], distinguished according to age at onset, but there seemed to be no association between the efficacy of bezafibrate and the age at onset in the present study.

It is not clear from our study whether bezafibrate is useful for improving the prognosis of PSC, because the study was retrospective, the sample size was small, and the follow-up period was limited. There was no defined study protocol because of the retrospective nature of this study. We could not describe the long-term effect of bezafibrate and could not analyze statistically the factors associated with the efficacy of bezafibrate. However, our report may be noteworthy in that the number of cases in our study was the same as the total number previously reported, and in that we reported the possibility of predicting the long-term effect of bezafibrate from the laboratory data 6 months after the initiation of its administration. Moreover, we discussed the relationship between the effectiveness of bezafibrate and liver function for the first time, albeit in a nonstatistical way.

In conclusion, bezafibrate can lower levels of hepatobiliary enzymes, especially as the first medical therapy in PSC. Further studies with a larger subset of patients and a long-term follow-up period are needed to evaluate the effect of this agent on the prognosis of PSC and to determine the prognostic factors for its efficacy.

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Expression of Pituitary Tumor–Transforming Gene 1 (PTTG1)/Securin in Hepatitis B Virus (HBV)-Associated Liver Diseases: Evidence for an HBV X Protein–Mediated Inhibition of PTTG1 Ubiquitination and Degradation

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Chronic infection with hepatitis B virus (HBV) is strongly associated with hepatocellular carcinoma (HCC), and the viral HBx protein plays a crucial role in the pathogenesis of liver tumors. Because the protooncogene pituitary tumor–transforming gene 1 (PTTG1) is overexpressed in HCC, we investigated the regulation of this protein by HBx. We analyzed PTTG1 expression levels in liver biopsies from patients chronically infected with HBV, presenting different disease stages, and from HBx transgenic mice. PTTG1 was undetectable in biopsies from chronic hepatitis B patients or from normal mouse livers. In contrast, hyperplastic livers from transgenic mice and biopsies from patients with cirrhosis, presented PTTG1 expression which was found mainly in HBx-expressing hepatocytes. PTTG1 staining was further increased in HCC specimens. Experiments *in vitro* revealed that HBx induced a marked accumulation of PTTG1 protein without affecting its messenger RNA levels. HBx expression promoted the inhibition of PTTG1 ubiquitination, which in turn impaired its degradation by the proteasome. Glutathione S-transferase pull-down and co-immunoprecipitation experiments demonstrated that the interaction between PTTG1 and the Skp1–Cul1–F-box ubiquitin ligase complex (SCF) was partially disrupted, possibly through a mechanism involving protein–protein interactions of HBx with PTTG1 and/or SCF. Furthermore, confocal analysis revealed that HBx colocalized with PTTG1 and Cul1. We propose that HBx promotes an abnormal accumulation of PTTG1, which may provide new insights into the molecular mechanisms of HBV-related pathogenesis of progressive liver disease leading to HCC development. (HEPATOLOGY 2010;51:777-787.)

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide.[1] Chronic infection with hepatitis B virus (HBV) is the main causal factor for HCC.[1] A growing body of evidence suggests that HBV may have a direct oncogenic capacity and that expression of virally encoded proteins, in particular the HBV X protein (HBx), promotes cell growth and tumor development.[2] Importantly, HBx expression is retained after viral integration into hepatocyte DNA[3] and is one of the most prevalent virus antigens in the liver and tumors of HBV carriers, and may induce humoral and cellular immune responses.[2] HBx alters sev-

Abbreviations: Ab, antibody; CFP, cyan fluorescent protein; Dox, doxycycline; GFP, green fluorescent protein; HA, hemagglutinin; HBV, hepatitis B virus; HBx, hepatitis B virus X protein; HCC, hepatocellular carcinoma; mRNA, messegner RNA; OA, okadaic acid; PP2A, protein phosphatase 2A; PTTG1, pituitary tumor-transforming gene 1; RT-PCR, reverse-transcription polymerase chain reaction; SCF, Skp1–Cul1–F-box ubiquitin ligase complex; siRNA, small interfering RNA.

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eral host functions that may lead to the carcinogenic process, including cell proliferation, viability, DNA repair, and genome stability.[2] Although HBx does not bind directly to DNA, it may activate the transcription of a wide range of cellular genes by different mechanisms involving activation of signal transduction pathways or direct interaction with components of the transcriptional machinery.[2] Recently, it has been proposed that HBx may also alter gene expression by promoting epigenetic changes in the DNA methylation profile[4] or by enhancing the stability of transcription factors such as HIF-1α[5] and c-myc.[6] Thus, HBx expression results in transcriptional activation of a variety of cellular genes involved in inflammation, angiogenesis, fibrosis, oxidative stress, and tumor development and progression.[2]

Pituitary tumor–transforming gene 1 (PTTG1)-encoded protein, originally isolated from pituitary tumor cells,[7] was later identified as a human securin, a protein implicated in inhibition of sister chromatid separation during mitosis, which has been associated with malignant transformation and tumor development.[8] Furthermore, PTTG1 plays key roles in cellular growth, DNA repair, development, and metabolism.[9] Mechanisms of PTTG1 action include protein–protein interactions, transcriptional activity, and paracrine/autocrine regulation.[9] During mitosis and following chromosome alignment, PTTG1 is degraded by the proteasome at metaphase to anaphase transition through the anaphase-promoting complex/cyclosome, releasing inhibition of separase, which in turn mediates the proteolysis of the cohesins ring that holds sister chromatids together.[8] In nonmitotic cells, the Skp1–Cul1–F-box protein ubiquitin ligase complex (SCF) is involved in the degradation of phosphorylated forms of PTTG1.[10] Furthermore, the SCF complex is involved in PTTG1 turnover in cycle-arrested cells after ultraviolet radiation.[11] PTTG1 overexpression has been reported in a great variety of tumors in which it correlates with invasiveness,[9] and it has been identified as a key signature gene associated with tumor metastasis.[12] In HCC, PTTG1 is overexpressed, and its expression levels have prognostic significance for the survival of postoperative HCC patients.[13] Interestingly, it has been proposed that PTTG1 might be critically involved in the development of HCC through the promotion of angiogenesis.[13] PTTG1 specifically interacts with p53, both *in vitro* and *in vivo*, and inhibits the ability of p53 to induce cell death, demonstrating its oncogenic potential.[14] Additionally, PTTG1 overexpression in hepatoma cell lines negatively regulates the ability of p53 to induce apoptosis.[15] Considering that HBV infection and HBx protein are associated with HCC and that a relationship between PTTG1 expression levels and HCC exists, we analyzed whether HBx may alter PTTG1 expression in chronic HBV-infected patients, HBx transgenic mice, and HBV-containing or HBx-expressing cell lines to provide new insights into our understanding of the molecular pathogenic mechanisms of advanced liver disease associated with HBV chronic infection.

Patients and Methods

Patients, Transgenic Mice, and Immunohistological Assays. Fifteen patients with HBV-related chronic liver disease (five with chronic hepatitis, five with cirrhosis, and five with HCC) were included. HBx transgenic mice were derived by microinjection the HBx gene into fertilized eggs of CD-1 mice.[16] Immunohistological assays were performed by standard procedures.

Cell Culture. Chang liver, Chang liver pX-34 (p34x), AML12 4p and AML12 4pX cells (4pX) were grown as described.[17,18]

Plasmid and Transfections. The indicated expression vectors were transfected employing Lipofectamine Transfection Reagent according to the manufacturer's instructions.

Western Blot Analysis. Proteins were extracted and immunobloted using the indicated antibodies.

Cell Cycle Analysis. Growth profiles of propidium iodide–labeled cells were analyzed by means of flow cytometry.

Real-Time Quantitative Reverse-Transcription Polymerase Chain Reaction Analysis. RNA extraction

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and quantitative reverse-transcription polymerase chain reaction (RT-PCR) were performed as described.[19]

Detection of PTTG1 Ubiquitination, Coimmunoprecipitation and Pull-Down Assays. Cleared lysates were subjected to immunoprecipitation with the indicated antibodies. The immunocomplexes were captured with protein A-sepharose. GST proteins were expressed in *Escherichia coli*, purified with glutathione-sepharose 4B, and incubated with cellular extracts. In both assays, bound proteins were analyzed by means of western blotting.

Immunofluorescence Analysis and Confocal Microscopy. Cells were grown on coverslips and processed as described.[19]

Small Interfering RNAs and Transfections. Cells were transfected with 100 nM ON-TARGET plus SMARTpool small interfering RNAs (siRNAs) directed against human Cul1 or a nonspecific control siRNA.

A detailed description of the protocols and reagents employed is provided in the Supporting Materials and Methods.

Results

PTTG1 Expression Level Increases in HBx-Immunoreactive Cells as Chronic Hepatitis B Progresses to Cirrhosis and HCC. We first investigated the expression of PTTG1 and HBx in human liver biopsies during HBV-related hepatocarcinogenesis by staining serial liver sections with anti-PTTG1 and anti-HBx antibodies (Abs). In specimens from patients with chronic hepatitis B and weak HBx expression, PTTG1 was not detected in hepatocytes (Fig. 1A). As chronic liver disease progressed from chronic hepatitis B to cirrhosis, PTTG1 protein appeared in HBx-immunoreactive hepatocytes (Fig. 1A). PTTG1 staining increased in HCC specimens showing high HBx expression (Fig. 1A). Double immunofluorescence studies in HCC specimens revealed that the distribution of PTTG1 fit well with the pattern shown by HBx immunolabeling (Fig. 1B).

PTTG1 Expression Level Increases as HBx Transgenic Mouse Livers Progress to Hyperplasia and HCC. HBx is considered one of the most important determinants of HBV-induced hepatocarcinogenesis. We further investigated the expression of PTTG1 and HBx during HBx-induced hepatocarcinogenesis in HBx transgenic mouse livers. Beginning at the age of 2 months, HBx transgenic mouse liver showed centrilobular foci of cellular alteration with cytoplasmic vacuolation surrounding the central veins where hepatocytes with increased DNA synthesis were detected.[16] PTTG1 and HBx were not detected in nontransgenic normal mouse livers. In hyperplastic HBx-transgenic mouse livers, expression of PTTG1 was found mainly in the cytoplasm of hepatocytes in the centrilobular region, and distribution of PTTG1 was similar to that of HBx (Fig. 2). Strong expression of both PTTG1 and HBx was observed diffusely in HCC specimens (Fig. 2). Double immunofluorescence studies in transgenic mouse–derived HCC specimens confirmed that PTTG1 and HBx are coexpressed in cancer cells (Supporting Fig. 1).

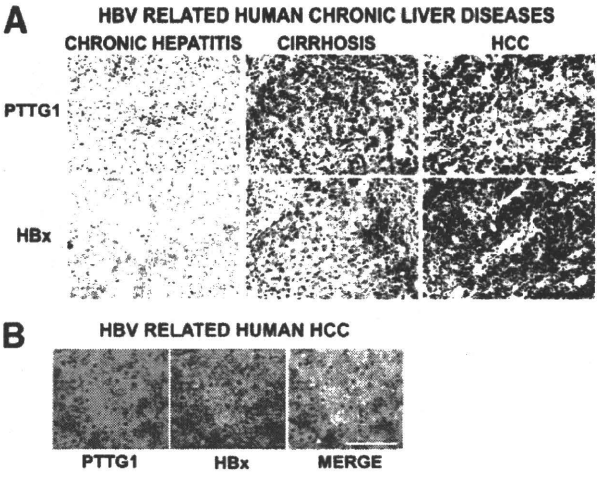

Fig. 1. PTTG1 expression levels increase as human chronic hepatitis B progresses to cirrhosis and HCC. (A) PTTG1 and HBx proteins were detected in human cirrhotic liver and HCC liver biopsies. PTTG1 protein appeared in HBx-immunoreactive hepatocytes in cirrhotic liver, and both proteins were strongly expressed in HCC specimens. Formalin-fixed, paraffin-embedded liver sections were stained with anti-PTTG1 Ab and anti-HBx. The HBx section was paired with an adjacent section stained using anti-PTTG1 Ab. Abs were then bound by goat anti-rabbit immunoglobulin G or by goat anti-mouse immunoglobulin G conjugated with peroxidase-labeled polymer. Peroxidase activity was detected using 3,3'-diaminobenzidine tetrahydrochloride. All sections were counterstained with hemotoxylin (blue). Brown coloring indicates specific Ab reactivity. We investigated five specimens of each chronic hepatitis B, cirrhosis, and HCC. Because HBx staining was not homogenous throughout the liver specimens of HCC and cirrhosis, selected fields with HBx expression are shown. Bar = 50 μm. (B) Hepatocytic PTTG1 in human HCC specimens colocalized with HBx. Human HCC sections were stained for immunofluorescence to simultaneously detect PTTG1 (red) and HBx (green). Yellow color indicates overlap of proteins. Bar = 50 μm.

HBx Expression Induces PTTG1 Accumulation. Because PTTG1 expression was increased during both HBV- and HBx-related chronic liver disease progression, we speculated that HBV and more precisely HBx might induce PTTG1 expression. We first examined whether a HBV replicon could induce PTTG1 expression. We transfected the hepatic-derived Chang liver cells with the plasmid payw1.2, which harbors 1.2 mer of the HBV genome that functions as an HBV replicon, and then evaluated PTTG1 expression by means of western blotting. The complete replicon induced the expression of

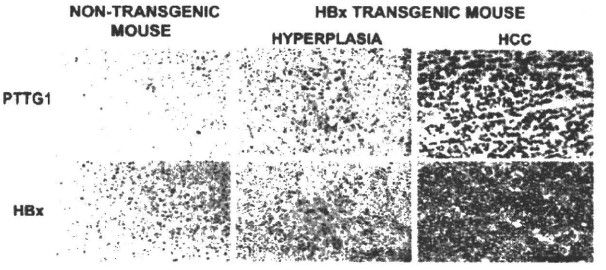

Fig. 2. PTTG1 oncoprotein increases as HBx transgenic mouse livers progress through hyperplasia to HCC. The distribution of PTTG1 and HBx in normal nontransgenic mouse liver, hyperplasia, and HCC specimens from HBx-transgenic mice is shown. PTTG1 was present mainly in the cytoplasm of hyperplastic hepatocytes immunoreactive for HBx oncoprotein surrounding central veins in HBx-transgenic mouse liver. Strong staining of both proteins was observed diffusely in HCC specimens. Immunohistochemical analyses were performed as described above. We investigated five biopsies each of normal, hyperplastic, and HCC liver. Because all of the results were similar among the experiments, representative results are displayed.

PTTG1 protein (Fig. 3A). Interestingly, PTTG1 expression in cells transfected with the HBx-defective whole-genome construct (payw*7) remained unchanged, indicating a role of HBx in PTTG1 induction (Fig. 3A). To further explore the effects of HBx on PTTG1 expression, we employed two hepatocyte-derived cell lines, Chang liver p34X (p34X) and AML12 4pX (4pX), in which HBx expression was controlled by doxicycline treatment (Dox-on) or withdrawal (Dox-off), respectively. Western blot analysis revealed increased PTTG1 expression upon induction of HBx over 48 hours in both Dox-regulated systems (Fig. 3B). Similar results were obtained after 24 hours of Dox treatment (Supporting Fig. 2A). As controls, we included Chang liver and AML12 4p cells—the parental cell lines of p34X and 4pX cells, respectively—and no PTTG1 variation after Dox challenge was observed. PTTG1 levels positively correlate with cell proliferation, and its expression is controlled in a cell cycle–dependent manner.[20] Several studies have also shown that HBx promotes cellular proliferation by triggering DNA synthesis and speeding up cell cycle progression.[21,22] However, evidence regarding the effects of HBx on liver cell proliferation and cell death is controversial, depending on the experimental systems and cell lines employed.[23] To assess the effect of HBx expression on cell cycle progression, we analyzed the growth profiles of Chang liver p34X and AML12 4pX cells with or without Dox treatment by means of flow cytometry. In agreement with previous reports,[24] our data showed that the percentages of p34X cells in G0/G1, S, and G2/M phases of the cell cycle displayed similar profiles 24 hours (Supporting Fig. 2B) and 48 hours (Fig. 3D) after induction of HBx expression. Furthermore, 4pX cells displayed a significant increase in HBx-dependent S phase entry 24 hours (Supporting Fig. 2B)[17] but not 48 hours (Fig. 3D) after induction of HBx expression. Additionally, transient transfection of Chang liver cells with the HBV wild-type and HBx-defective replicons did not induce changes in the cell cycle profile (Fig. 3C). Given that HBx promoted PTTG1 accumulation without significantly affecting cell

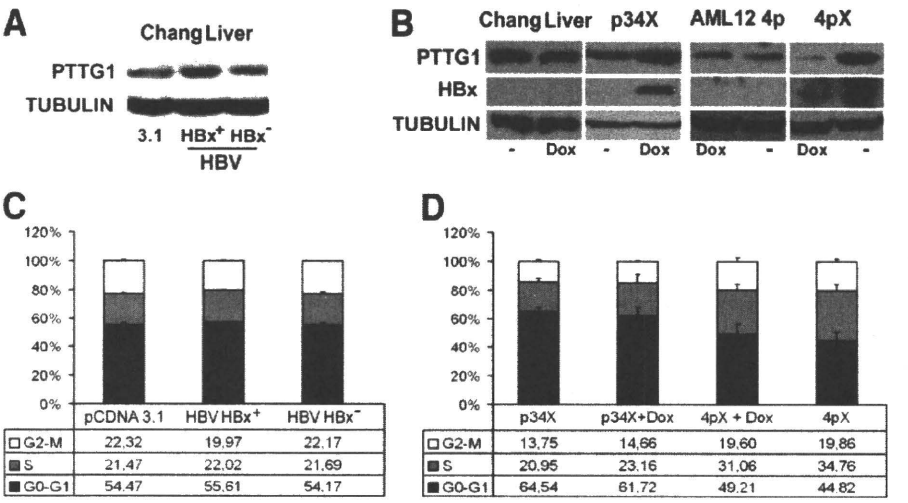

Fig. 3. Effects of HBx on PTTG1 expression. (A) Chang liver cells were transfected with the whole HBV genome payw1.2 (HBx+), an HBx-defective mutant payw7* (HBx−), or control plasmid (pcDNA 3.1), and PTTG1 expression was monitored by means of western blotting. Tubulin expression was assessed to ensure equal protein loading of all samples. (B) PTTG1 protein levels were analyzed in Chang liver, Chang liver p34X, AML12 4p, and AML12 4pX cells grown with or without Dox for 48 hours by means of western blotting. (C) Flow cytometry analysis of cell cycle progression in Chang liver cells 24 hours after transient transfection with payw1.2(HBx+), payw*7(HBx−), or control plasmid (pCDNA3.1). (D) Cell cycle progression in p34X and 4pX cells 48 hours after induction of HBx expression. Values represent the mean ± standard deviation of six independent experiments.

cycle (p34X and HBV complete replicon-transfected Chang liver cells), these results indicated that the HBx-promoted PTTG1 accumulation was not dependent on cell cycle modifications.

HBx-Mediated PTTG1 Accumulation Is Regulated Posttranscriptionally. It is known that HBx transcriptionally induces the expression of viral and cellular genes by activating promoter regulatory sequences.[2] To determine whether HBx modulates PTTG1 transcription, its messenger RNA (mRNA) levels were measured by means of quantitative RT-PCR in p34x and 4pX cells. PTTG1 mRNA levels were unaffected by HBx expression in both p34X (Fig. 4A) and 4px (Supporting Fig. 3) cells. As expected,[25] RT-PCR analysis revealed increased TNF-α mRNA levels upon induction of HBx (Fig. 4A).

Additionally, we transiently transfected Hela cells with both pPTTG1–cyan fluorescent protein (CFP), an expression vector in which PTTG1-CFP transcription is controlled by the CMV promoter, and pHBx-hemagglutinin (HA) plasmids. Western blot analysis using an anti–green fluorescent protein (GFP) Ab revealed that PTTG1-CFP was clearly accumulated in HBx-transfected cells (Fig. 4B). Interestingly, the effect of HBx was not observed when cells were cotransfected with the control plasmid pECFP-N1, coding only for the CFP protein. These results were further confirmed by cotransfecting Hela cells with wild-type or HBx-defective HBV replicons along with the pPTTG1-CFP vector (Fig. 4C). These results strongly suggested that PTTG1 accumulation induced by HBx was not mediated by transcriptional activation.

We next examined whether HBx-induced PTTG1 upregulation could be explained through changes on protein stability by analyzing PTTG1 levels after blocking protein synthesis with cycloheximide. Western blot analysis revealed that PTTG1 protein half-life increased in p34X cells after induction of HBx expression when compared with noninduced cells (Fig. 4D,E). Taken together, these results indicated that HBx promoted PTTG1 accumulation by modulating its degradation.

HBx Inhibits the Ubiquitination of Hyperphosphorylated PTTG1 Forms. Phosphorylation of PTTG1

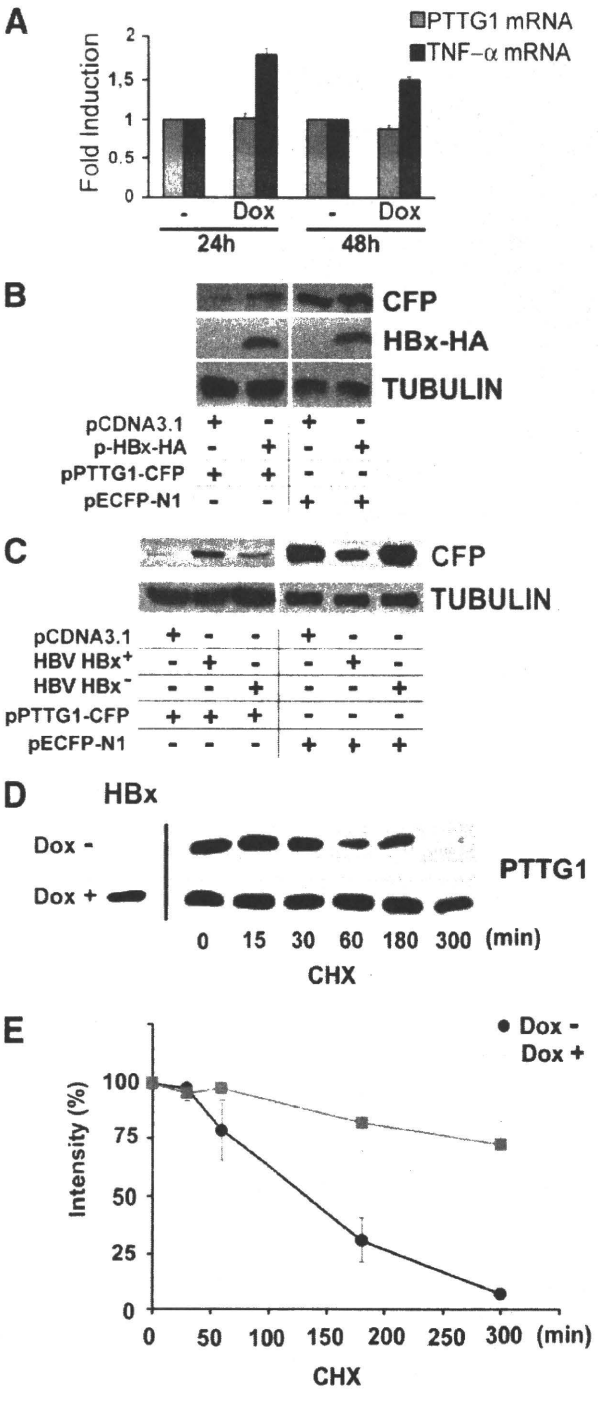

Fig. 4. HBx increases PTTG1 expression levels posttranscriptionally. (A) PTTG1 (light gray bars) and TNF-α (dark gray bars) mRNA levels were measured in p34X cells 24 and 48 hours after induction of HBx expression by means of quantitative RT-PCR. Results were normalized with histone H3 and represented as fold induction over control. Results are expressed as the mean ± standard deviation of three independent experiments. (B) Hela cells were transfected with pPTTG1-CFP or pECFP-N1 plus pHBx-HA or empty vector (pcDNA 3.1). Cell lysates were analyzed by means of western blotting. (C) Hela cells were transfected with pPTTG1-CFP or pCFP-N1 plus payw1.2, payw*7, or control plasmid. Cell lysates were analyzed by means of western blotting. (D) Control or Dox-induced p34x cells (48 hours) were treated with 20 μM cycloheximide for different time intervals. Equal amounts of protein were subjected to Western blot analysis. Representative results of three independent experiments are shown.(E) Analysis of PTTG1 relative levels, assessed by scanning densitometry, was plotted (black circles, control cells; gray squares, HBx-expressing cells). Results are expressed as the percentage of values obtained without cycloheximide treatment for each experimental condition analyzed. Values represent the mean ± standard error of three independent experiments.

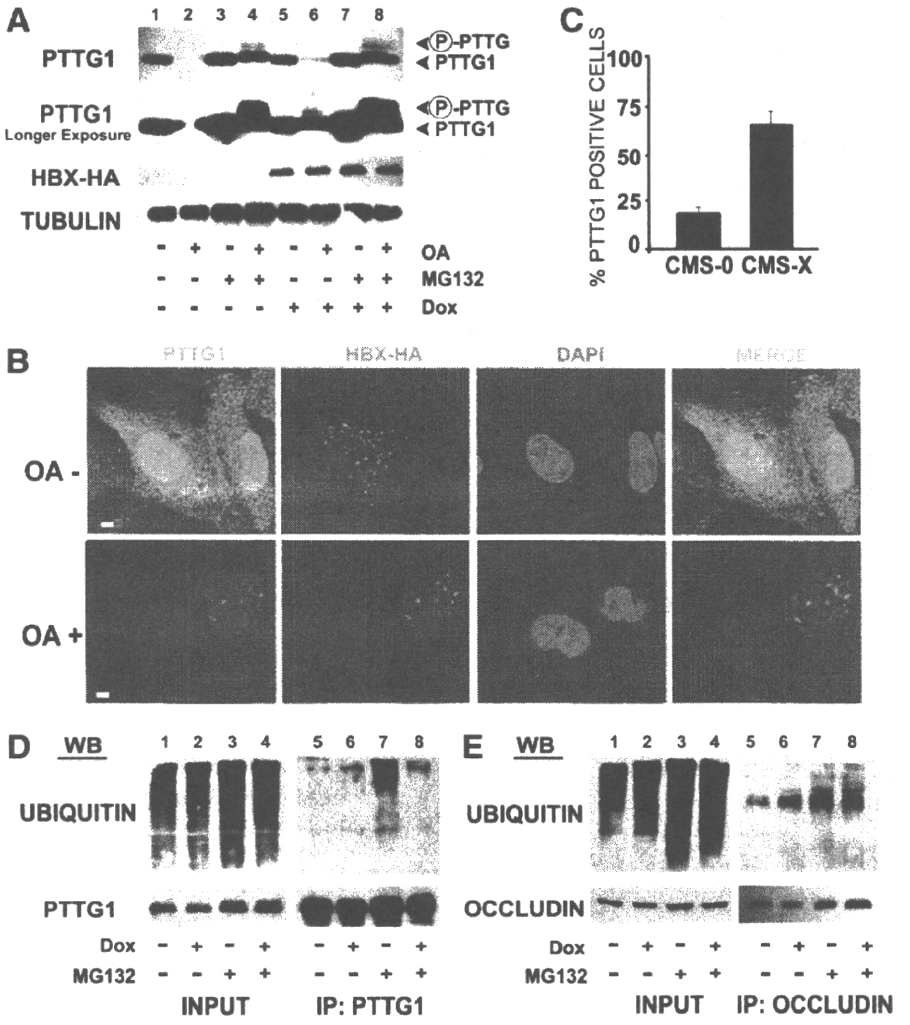

Fig. 5. HBx reduces the ubiquitination of hyperphosphorylated PTTG1 forms. (A) Chang liver p34x cells were treated with OA (1 μM), MG132 (10 μM), or both for 2 hours after 48 hours of Dox induction. Lysates were subsequently analyzed by means of western blotting for the detection of PTTG1. (B) Confocal immunofluorescence analysis of the distribution of PTTG1 (green) and HBx (red) in OA-treated (OA+) or nontreated (OA−) p34x cells. 4′.6-Diamidino-2-phenylindole staining is shown in blue. Bar = 5 μm. (C) Twenty-four hours after transfection of Chang liver cells with pCMS-EGFP-HBx (CMS-X) or pCMS-EGFP (CMS-0) plasmids, cells were treated with OA, and PTTG1 accumulation was compared by means of immunofluorescence. Percentages of transfected cells (GFP positive) displaying PTTG1 accumulation are shown. Values are expressed as the mean ± standard deviation of four independent experiments in which at least 50 GFP-positive cells were analyzed. (D) Control or Dox-induced p34X cells were treated or not for 4 hours with MG132 (10 μM). Cell lysates were immunoprecipitated (IP) with anti-PTTG1 pAb and immunoblotted with anti-PTTG1 (bottom) and anti-ubiquitin (top) Abs. (E) Cells were treated as in (D), and lysates were immunoprecipitated with anti-occludin pAb and immunoblotted with anti-occludin (bottom) and anti-ubiquitin (top). Representative results of at least two independent experiments are shown.

leads to its ubiquitination and proteasomal degradation.[10] Thus, we analyzed the levels of phosphorylated forms of PTTG1 in p34X cells treated with okadaic acid (OA), a protein phosphatase 2A (PP2A) inhibitor, and/or MG132, a proteasome inhibitor. As expected, proteasome inhibition by MG132 treatment promoted PTTG1 accumulation independently of HBx expression (Fig. 5A, lane 3 versus lane 1 and lane 7 versus lane 5). As described,[10] MG132 plus OA cotreatment revealed the presence of slower migrating bands corresponding to phosphorylated forms of PTTG1 in both control or Dox-treated cells (Fig. 5A, lanes 4 and 8). OA treatment reduced PTTG1 levels in both HBx-expressing and -nonexpressing cells (Fig. 5A, lane 2 versus lane 1 and lane 6 versus lane 5). However, phosphorylated PTTG1 could be detected in the absence of MG132 after PP2A inhibition (OA treatment) only when HBx was expressed, suggesting that HBx inhibited the degradation of phosphorylated PTTG1 (Fig. 5A, lane 6 versus lane 2). In order to rule out that the differences observed between HBx-expressing and -nonexpressing cells could be due to undefined clonal properties of p34X cells or Dox-associ-