APE1/Ref-1 enhances DNA binding activity of mutant p53 in a redox-dependent manner

YANPING CUN[1*], NAN DAI[1*], MENGXIA LI[1], CHENGJIE XIONG[2], QINHONG ZHANG[1], JIANGDONG SUI[1], CHENGYUAN QIAN[1] and DONG WANG[1]

[1]Cancer Center, Daping Hospital and Research Institute of Surgery, Third Military Medical University, Chongqing; [2]Department of Orthopedics, Wuhan General Hospital, Guangzhou Military Area Command, Wuhan, P.R. China

Abstract. Apurinic/apyrimidinic endonuclease 1/redox factor-1 (APE1/Ref-1) is a dual function protein; in addition to its DNA repair activity, it can stimulate DNA binding activity of numerous transcription factors as a reduction-oxidation (redox) factor. APE1/Ref-1 has been found to be a potent activator of wild-type p53 (wtp53) DNA binding *in vitro* and *in vivo*. Although p53 is mutated in most types of human cancer including hepatocellular carcinoma (HCC), little is known about whether APE1/Ref-1 can regulate mutant p53 (mutp53). Herein, we reported the increased APE1/Ref-1 protein and accumulation of mutp53 in HCC by immunohistochemistry. Of note, it was observed that APE1/Ref-1 high-expression and mutp53 expression were associated with carcinogenesis and progression of HCC. To determine whether APE1/Ref-1 regulates DNA binding of mutp53, we performed electromobility shift assays (EMSAs) and quantitative chromatin immunoprecipitation (ChIP) assays in HCC cell lines. In contrast to sequence-specific and DNA structure-dependent binding of wtp53, reduced mutp53 efficiently bound to nonlinear DNA, but not to linear DNA. Notably, overexpression of APE1/Ref-1 resulted in increased DNA binding activity of mutp53, while downregulation of APE1/Ref-1 caused a marked decrease of mutp53 DNA binding. In addition, APE1/Ref-1 could not potentiate the accumulation of p21 mRNA and protein in mutp53 cells. These data indicate that APE1/Ref-1 can stimulate mutp53 DNA binding in a redox-dependent manner.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer-related mortality worldwide. It has a five year natural mortality rate of >95%,

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Correspondence to: Professor Dong Wang, Cancer Center, Daping Hospital and Research Institute of Surgery, Third Military Medical University, Chongqing, P.R. China
E-mail: dongwang64@hotmail.com

*Contributed equally

and it affects >500,000 people in the world each year; >50% of the new HCC cases and deaths have occurred in China (1). Despite considerable efforts to improve the survival of patients with HCC, a satisfactory level has not been achieved as only 15% of the patients are eligible for optimal resection at diagnosis and the tumor cells exhibit an inherent tumor chemo- and radioresistance.

DNA-repair systems, as the molecular basis of defending against environmental damage to cell DNA, play an important role in maintaining the genomic stabilization and integrity. However, an elevated DNA repair capacity in tumor cells leads to drug or radiation resistance and severely limits the efficacy of chemotherapy and radiotherapy. Apurinic/apyrimidinic endonuclease 1/redox factor-1 (APE1/Ref-1; APE1) is an essential enzyme in the DNA base excision repair (BER) pathway, which plays a critical role in the repair of DNA caused by oxidative and alkylation damage (2). APE1 accounts for 95% of the abasic site cleavage activity in human cells, and is essential for the protection of cells against the toxic effects of endogenous and exogenous agents. In addition to its DNA repair functions, APE1 participates in other crucial cellular processes, including the response to oxidative stress and regulation of some transcriptional factors, including p53 (3). Moreover, several studies demonstrated that APE1 was high-expressed in several human tumors including prostate, osteosarcomas, lung and cervical carcinoma, and the elevated APE1 level was associated with chemo- and radioresistance and poor clinical outcome (4,5). Silencing of APE1 enhanced cell sensitization to chemotherapeutic agents and radiation (6,7). In other types of cancer, the shift of APE1 from nucleus to cytoplasm was observed compared with normal tissues, and might play a pivotal role in carcinogenesis and progression of several human tumors (8,9). Tumor cells often show increased expression level and altered subcellular localization of APE1, and are associated with chemo- and radioresistance and tumor carcinogenesis and progression.

The tumor suppressor gene p53 is activated in response to DNA damage and induces cell cycle arrest or apoptosis, and thus helps to maintain genomic stability and prevent cancer (10,11). Once p53 is activated, it induces cell apoptosis by both transcription-dependent and -independent mechanisms, or arrests cell cycle by transactivation of Waf-1/p21, 14-3-3σ. p53 is one of the most commonly mutated genes in

human cancer; more than half of all types of human cancer contain mutant or inactive p53. The mutant p53 (mutp53) proteins not only lose their tumor suppressive activities but often gain additional oncogenic functions (12). p53 mutations are detected in human HCC and its inactivation is correlated with chemo- and radioresistance, and the carcinogenesis and progression of HCC (13,14).

APE1 as a redox regulator is responsible for reducing wild-type p53 (wtp53), thus enhancing its DNA-binding activity by redox-dependent and -independent mechanisms (15,16). Moreover, the redox-independent activation of wtp53 is due to a regulatory interaction of APE1 with the p53 C-terminal regulatory domain (CRD) (15), which is intact in most of the frequently encountered mutp53 proteins, containing the most common R249S mutation in HCC. Although mutp53 proteins have lost the sequence-specific DNA binding (SSDB) transcriptional activity, they retained the potential to bind nonlinear DNA in a DNA structure-dependent manner (17). Given the activation of wtp53 by APE1 and the intact CRD in mutp53, we propose that APE1 may also regulate the DNA binding activity of mutp53. Elucidating the combined expression of APE1 and p53 in human cells may be of major clinical significance, and a clear understanding of the mechanisms by which APE1 controls mutp53 expression may aid in the effective use of chemotherapeutic agents or multigene therapy strategies in the treatment of tumors.

In this study, we first investigated the expression level and subcellular localization of APE1, and its correlation with p53 expression and clinicopathological parameters in HCC. Then, we performed electromobility shift assay (EMSA) and quantitative chromatin immunoprecipitation assay (ChIP) to determine whether APE1 can regulate mutp53 DNA binding activity. We showed that the increased APE1 expression level was significantly correlated with p53 expression, and the high APE1 expression/p53[+] status indicated a higher tumor grade. We also present evidence that APE1 enhanced reduced mutp53 binding to the nonlinear DNA in a redox-dependent manner. In addition, APE1 could not increase p21 mRNA and protein levels in mutp53 cells. To our knowledge, this is the first direct evidence to show that APE1 stimulates mutp53 DNA binding activity, and these findings provide new insights into the functional linkage between APE1 and p53 in cancer therapy.

Materials and methods

Patients and tissues. Tumor tissues were obtained from 103 patients with hepatoma at the Department of Cancer Center, Daping Hospital, Third Military Medical University, China, from 1991 to 2004. The local ethics committee approved this study. No chemotherapy or radiotherapy was administered to patients prior to surgery. Histological grading according to Edmondson and Steiner's standard: grade I, 5 cases; grade II, 27 cases; grade III, 56 cases and grade IV, 15 cases. In HCC cases, 40 liver samples were sufficiently large to include both the tumor and the surrounding cirrhosis. Ten patients who underwent resection of hepatic angioma were used as controls.

Immunohistochemistry and APE1 scoring. The expression of APE1 protein was analyzed using immunohistochemistry. Sections from paraffin-embedded tumors were incubated overnight with mouse anti-human APE1 monoclonal antibody (Novus Biologicals, Littleton, CO, USA) at a 1:2,000 dilution and anti-p53 antibody (DO-1) (Santa Cruz Biotechnologies, Inc., Santa Cruz, CA, USA) at a 1:500 dilution, and then incubated with goat anti-mouse secondary antibody from Pierce (Rockford, IL, USA). Antigen-antibody complexes were visualized by incubation with 3,3'-diaminobenzidine (DAB) substrate and counterstained with diluted Harris hematoxylin. Tissues were scored for: i) percentage of cell staining and ii) intensity of staining (low, moderate, or high). To be defined as low expression, the tissue needed to meet weak staining and positive cell percentage <50% or moderate staining and positive percentage <25%.

Cell culture. Human hepatoma cell lines HepG2 (harboring wtp53), Hep3B (P53 null) and MHCC97L (carrying mutp53) were obtained from the Cell Institute of Shanghai (Academia Sinica, Shanghai, China). Cells were maintained as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 50 mg/ml penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Cells were grown at 37˚C in a humidified incubator under 5% CO_2.

Infections. Adenovirus vector Ad5/F35-siAPE1 carrying human APE1 siRNA sequence was constructed by Xiang *et al* (7). The control adenovirus, Ad5/F35-EGFP, was purchased from Vector Gene Technology Co., Ltd. (Beijing, China). HepG2 and MHCC97L cell lines were infected with Ad5/F35-EGFP or Ad5/F35-siAPE1 for 2 h and then washed to remove the adenoviruses. Cells were cultured for another 48 h and then analyzed by western blotting or prepared for subsequent experiments. Cells were transfected with p3XFLAG-CMV/APE1, the wild-type (WT) APE1, using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol. As controls, cells were transfected with the p3XFLAG-CMV-14 empty vector (Sigma-Aldrich). At 24-h post-transfection, the transfected cells were transferred into normal growth medium. After a further 24 h, cells were prepared for subsequent experiments.

Western blot analysis. Equal amounts of nuclear or cytosolic extract or whole-cell lysate, obtained from HepG2, Hep3B and MHCC97L cells, were electrophoresed by 10% SDS-polyacrylamide gels. The proteins were then transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA) and blocked in Tris-Buffered Saline and Tween-20 (TBST) [50 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.1% (volume/volume) Tween-20] containing 5% (weight/volume) defatted milk for 1 h at room temperature. Membranes were incubated with the specific primary antibody. After three washes with TBST, the membranes were incubated for 1 h at room temperature with a horseradish peroxidase-conjugated secondary antibody (1:2,000) (Pierce). Then, the membranes were washed three times with TBST and the blots were reacted with chemiluminescence reagents and revealed with Biomax-Light films (Kodak, Rochester, NY, USA). Band intensities were analyzed using the Gel Doc 2000 apparatus and software (Quantity One; Bio-Rad). Suppliers of incubation conditions for antibodies used for western blotting were: anti-APE1
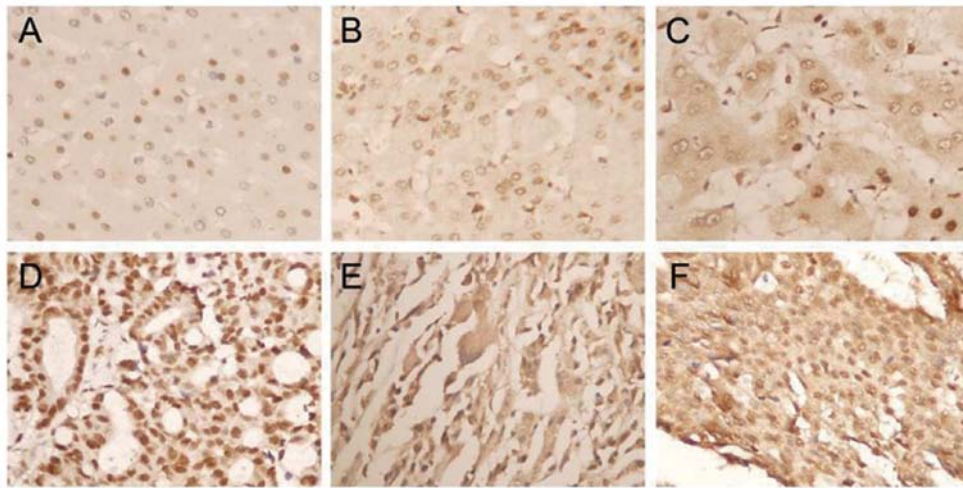
Figure 1. APE1 immunohistochemical staining. (A) Nuclear reactivity in normal tissues; (B) nuclear reactivity in liver cirrhosis tissues; (C) both nuclear and cytoplasmic reactivity in liver cirrhosis tissues; (D) nuclear reactivity in HCC tissues; (E) cytoplasmic reactivity in HCC tissues; (F) both nuclear and cytoplasmic reactivity in HCC tissues.

monoclonal (Novus Biologicals), 1 h at 37˚C, dilution 1:5,000; anti-p21 monoclonal (Santa Cruz Biotechnologies, Inc.), overnight at 4˚C, dilution 1:500; anti-p53 monoclonal (DO-1), overnight at 4˚C, dilution 1:500; anti-β-actin monoclonal (Santa Cruz Biotechnologies, Inc.), 1 h at 37˚C, dilution 1:2,000.

Electrophoretic mobility shift assays. Nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) according to the manufacturer's instructions. Electrophoretic mobility shift assays (EMSAs) were performed following the manufacturer's instruction manual of LightShift Chemiluminescent EMSA kit (Pierce). Briefly, 5 µg of nuclear extracts was incubated with 3'-biotin labeled and purified double-stranded oligonucleotide probe. The p53 response element of the *p21^{Waf1}*-promoter (p53-RE) was prepared either in its linear form (P53RE-linF, 5'-gctctgccGAACATGTCCCAACATGTTG ccgctctg-3' and P53RE-linR, 5'-cagagcggCAACATGTTG GGACATGTTCggcagagc-3') or in a stem loop conformation (P53RE-strF, 5'-ccgcggtaccattacctaaggcgtc-3' and P53RE-strR 5'-gacgccttaggtacctgccGAACATGTCCCAACATGTTgggcctg atggtaccgcgg-3') as previously described (18) (Invitrogen, Shanghai, China). Upper case letters indicate the p53-binding sites. Following incubation, samples were separated on a prerun 5% polyacrylamide gel at 100 V for 90 min and then transferred to a Zeta-Probe GT nylon membrane (Bio-Rad). The probes were detected by HRP-conjugated streptavidin (1:300) and the bands visualized by ECL reagents provided with the kit. The resultant bands were quantified using Quantity One imaging software (Bio-Rad).

Chromatin immunoprecipitation assays. Chromatin immunoprecipitation (ChIP) assays were performed to analyze the DNA binding affinity of p53 to the p21 promoter region of its downstream genes using a ChIP kit (Millipore) according to the manufacturer's instructions. Cells incubated in 10-cm petri dishes and subjected to various infection treatments were harvested and crosslinked with 1% formaldehyde. Pellets were collected and resuspended in ice-cold lysis buffer, and then the chromatin was broken down through ultrasonication to the DNA fragments at an average size of 200-500 bp, as empirically estimated by agarose gel electrophoresis. Immunoprecipitations were performed with p53 antibody (DO-1) to fractionate p53 protein-DNA complexes and IgG (as a negative control). Quantitative PCR was then performed to amplify the p21 gene promoter region (containing potential specific p53 binding sites) using primers: CCCTTCCTCACCTGAAAACA and GTGGCTCTGTTGGCTTTCTG. The predicted sizes of the products were 116 bp. Preimmunoprecipitation lysates were also included as input controls.

mRNA analysis by quantitative RT-PCR. Infected and control cells (1x10^6) were harvested and washed using cold PBS once. Total RNA was extracted by using TRIzol (Invitrogen) and chloroform/isoamyl alcohol precipitation following the manufacturer's instructions. RNA concentrations were determined by spectro-photometer (Eppendorf AG, Hamburg, Germany). Then, 1 µg of total RNA was reverse transcribed into single-stranded DNA by using SuperScript II (Invitrogen). Quantitative RT-PCR was performed using SYBR Premix Ex Taq (Takara) in a LightCycler 480 real-time PCR system (Roche, Indianapolis, IN, USA). Primers for p21 were: CTGG AGACTCTCAGGGTCGAAA and TTCTCCAAGAGGAA GCCCTAATC; and for control β-actin: GATCATTGCTCC TCCTGAGC and TGTGGACTTGGGAGAGGACT. Gene expression was determined by normalization against β-actin expression.

Statistical analysis. Associations between categorical groups (i.e., APE1 expression and clinicopathologic data, APE1 and p53 expression, APE1/p53 expression and tumor histologic grade) were examined using Chi-square analysis. All p-values were two sided, and p-values <0.05 were considered to indicate statistically significant differences. For ChIP assays, data were obtained from three independent experiments and expressed as mean ± standard deviation values, and then analyzed using the one-way ANOVA test with computer SPSS software SPSS 10.0 (SPSS, Chicago, IL, USA).

Table I. The relationship between clinicopathologic factors and APE1 protein expression in 103 hepatoma cases.

Clinicopathologic data	No. of cases	Nucleus expression			Cytoplasm expression		
		-	+	++	-	+	++
Age (years)							
<45.5	37	2	6	29	15	15	7
≥45.5	66	2	13	51	33	21	12
Gender							
Male	93	3	17	73	46	30	17
Female	10	1	2	7	3	6	1
Tumor size (cm)							
Single tumor ≤3	24	1	7	16	11	7	6
Single tumor 3-5	15	0	4	11	5	3	7
Single tumor ≥5, or ≥ two tumors	64	3	8	53	32	26	6
Serum HBsAg							
Positive	81	2	11	68	43	26	12
Negative	22	2	8	12	5	10	7
TNM stage							
II	38	2	5	31	10	20	8
IIIA	16	0	3	13	8	8	0
IIIB	2	0	0	2	0	2	0
IVA	36	1	8	27	21	6	9
IVB	11	1	3	7	9	0	2
Histologic grade							
Grade I-II	32	3	9	20	22	10	0
Grade III-IV	71	1	10	60[a]	26	26	19[b]

[a]p<0.05, [b]p<0.01 vs. Grade I-II.

Table II. Relationship between APE1/P53 expression and tumor grade malignancy.

Histologic grade	Total	APE1/P53 expression				P-value
		+/-	+/+	++/-	++/+	
Grade I-II	32	15	7	4	6	
Grade III-IV	71	16	8	6	41	
Total	103	31	15	10	47	<0.01

Results

APE1 immunohistochemistry and clinicopathologic parameters. We investigated the expression of APE1 in 10 normal liver tissues, 40 liver cirrhosis tissues and 103 cases of HCC tissues using immunohistochemistry. As shown in Fig. 1, APE1 staining was mainly located in the nucleus in normal liver tissues, and was located not only in the nucleus, but also in the cytoplasm in liver cirrhosis and HCC tissues. Twenty-five of 40 liver cirrhosis tissues (62.5%) were nucleus staining and 8 tissues (20%) showed both the nucleus and cytoplasm staining. Three subcellular locations of APE1 protein were observed, and were nucleus staining in 48 cases (46.6%), cytoplasm staining in 4 cases (3.9%) and staining in both the nucleus and cytoplasm in 51 cases (49.5%) in HCC tissues. Additionally, there was a significant difference in the cytoplasmic and nuclear staining intensity of APE1 among normal liver tissue, liver cirrhosis and HCC tissues. As shown in Table I, APE1 expression was not related to age, gender, tumor size, serum HBsAg and TNM stage, whereas there was a significant difference between the APE1 protein expression among patients with different histologic classification (Grade I-II vs. Grade III-IV).

Relationship between APE1/mutp53 expression and tumor grade malignancy. We also investigated the expression of p53 in 10 normal liver tissues, 40 liver cirrhosis tissues and 103 cases of HCC tissues using immunohistochemical assay. Immunohistochemical staining showed that p53 staining was mainly located in the nucleus in 60.2% (62/103) HCC tissues, and no p53 staining was detected in both normal liver tissues and liver cirrhosis tissues. Also, 44.66% (46/103) HCC tissues showed lower APE1 expression, and the remaining 55.34%
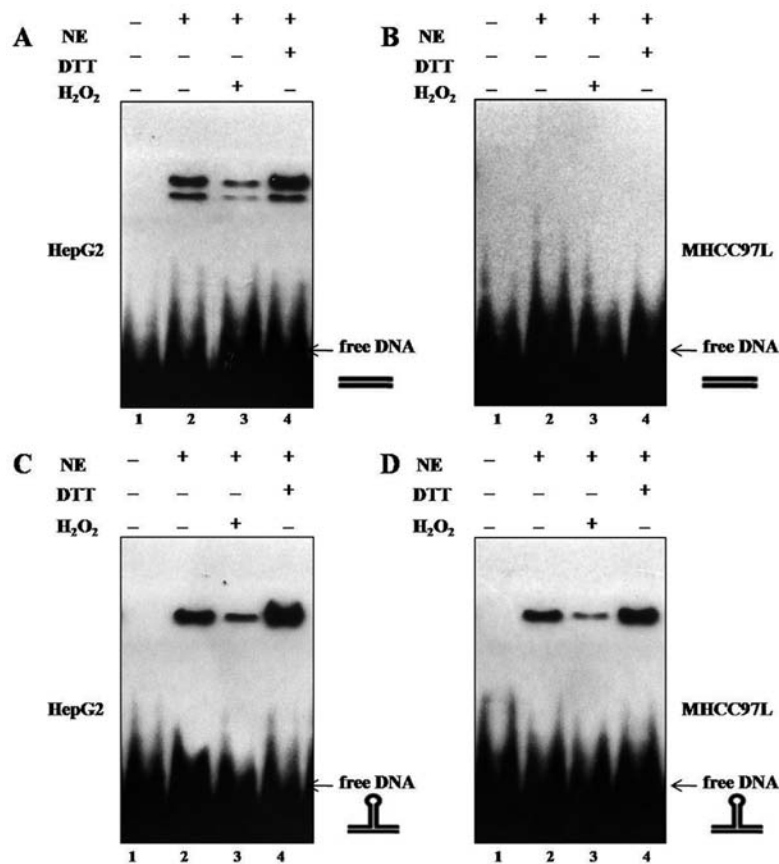
Figure 2. Transcriptional activation by active and latent forms of p53. Nuclear protein of HepG2 and MHCC97L cells was used in EMSA to analyze DNA binding to the p53 response element of the p21^{Waf1}-promoter in either a linear conformation, p53-RE$_{lin}$, or stabilized in a stem-loop structure, p53-RE$_{struct}$. (A) The wtp53 DNA binding to linear DNA is promoted by DTT and inhibited by H$_2$O$_2$. (B) Mutp53 protein did not bind to linear DNA. Reduction of wtp53 (C) and mutp53 (D) by DTT increases p53 DNA binding to nonlinear DNA. EMSA, electromobility shift assay.

(57/103) of tissues showed high APE1 expression. Using the Chi-square test, we found that the APE1 high expression was associated with mutp53 status.

As shown in Table II, we separated these HCC patients into Grade I-II (32 cases) and Grade III-IV (71 cases). According to APE1 expression level and p53 status, 103 HCC patients were separated into four groups: APE1$^+$/p53$^-$, APE1$^+$/p53$^+$, APE1^{++}/p53$^-$ and APE1^{++}/p53$^+$. The level of APE1 combination with the mutp53 status was significantly correlated with tumor grade, and APE1^{++}/p53$^+$ status indicated a higher tumor grade.

Mutant p53 binds strongly and specifically to nonlinear DNA. We selected the p53 response element of the p21^{waf1}-promoter (p53-RE) as target DNA to analyze the binding of human p53 protein. The p53-RE was prepared either in its linear form (p53-RE$_{lin}$) or in a stem loop conformation (p53-RE$_{struct}$) as previously described (18). As shown in Fig. 2A and C, we demonstrated that reduced wtp53 proteins bound efficiently to linear and nonlinear DNA, indicating that binding of wtp53 to either DNA conformation requires a reduced status of the p53-DBD, in accordance with previous reports (16,19,20). Compared to the high-affinity binding of wtp53, mutp53 bound efficiently to nonlinear DNA (Fig. 2D), but not to linear DNA (Fig. 2B), in line with the findings of Gohler *et al*, and the specific and selective binding of mutp53 to nonlinear DNA provide mutp53 proteins as DNA structure-specific

DNA-binding (DSSB) proteins (17). DTT application enhanced the mutp53 DNA binding to nonlinear DNA, whereas addition of H$_2$O$_2$ attenuated the binding of mutp53 to the nonlinear DNA substrate p53-RE$_{struct}$, indicating that binding of mutp53 to nonlinear DNA requires a reduced p53 status as well as in wtp53 DNA binding (16) (Fig. 2D). Thus, enhanced DNA binding to nonlinear DNA by addition of DTT corresponds to DNA binding by reduced mutp53.

APE1 stimulates mutant p53 DNA binding to nonlinear DNA. To test whether APE1 influences p53 DNA binding, cells were infected with p3XFLAG-CMV/APE1 (WTAPE1) or p3XFLAG-CMV-14 empty vector, Ad5/F35-APE1 siRNA or Ad5/F35-EGFP. We found that addition of the WTAPE1 further and strongly enhanced binding of wtp53 to p53-RE$_{lin}$ DNA (Fig. 3A) and p53-RE$_{struct}$ DNA (Fig. 3C) compared with p3XFLAG-CMV-14, as previously described by Jayaraman *et al* (15). Moreover, Ad5/F35-APE1 siRNA attenuated wtp53 DNA binding to either DNA conformation compared with Ad5/F35-EGFP (Fig. 3A and C). Of note, WTAPE1 enhanced binding of mutp53 to p53-RE$_{struct}$ DNA (Fig. 3D), but not to p53-RE$_{lin}$ DNA (3B). Additionally, Ad5/F35-siAPE1 attenuated p53 DNA binding to p53-RE$_{struct}$ compared with Ad5/F35-EGFP (Fig. 3D).

To further examine whether APE1 also influences p53 DNA binding independently of its reducing activities, we
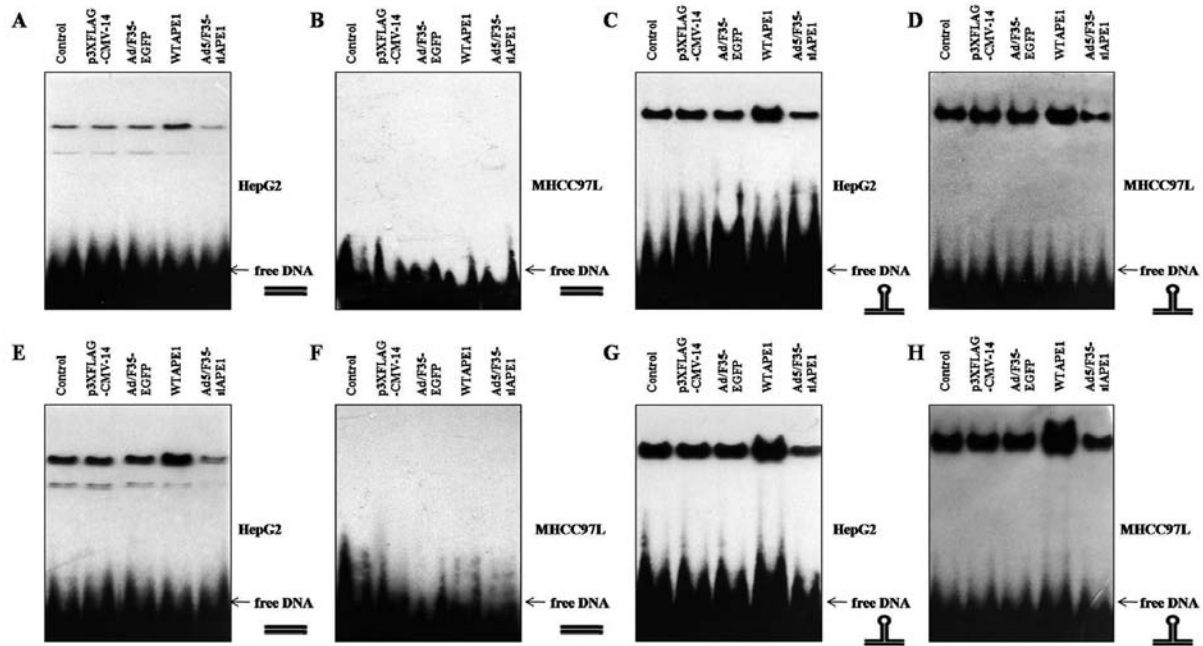
Figure 3. Effect of APE1 on p53 DNA binding activity by EMSA. (A) APE1 enhances binding of wtp53 to linear DNA, and downregulation of APE1 suppresses the DNA binding of wtp53 to linear DNA. (B) Mtp53 can not bind to linear DNA either in the WTAPE1 or the Ad5/F35-siAPE1 treatment group. APE1 enhances binding of wtp53 (C) and mtp53 (D) to nonlinear target DNA; meanwhile, silencing of APE1 decreases the DNA binding of wtp53 and mtp53 to nonlinear DNA. DNA binding of reduced p53 (pre-treatment by DTT) was analyzed by EMSA in the WTAPE1 and Ad5/F35-siAPE1 groups. (E) APE1 enhances reduced wtp53 DNA binding to linear DNA, while Ad5/F35-siAPE1 inhibits the DNA binding of reduced wtp53 to linear DNA. (F) Reduced mtp53 also did not bind to linear DNA either in the WTAPE1 or the Ad5/F35-siAPE1 group. APE1 effectively increases the DNA binding of reduced wtp53 (G) and mtp53 (H) to nonlinear DNA. EMSA, electromobility shift assay.

examined the effects of APE1 in the presence of DTT. As shown in Fig. 3E and G, WTAPE1 potentiated the wtp53 DNA binding to both linear and nonlinear DNA structures, and decreased APE1 attenuated the wtp53 DNA binding activities. We next examined the effects of APE1 on reduced mutp53 DNA binding and found that the reduced mutp53 also did not bind to linear DNA (Fig. 3F), and the binding of reduced mutp53 to nonlinear DNA was enhanced by WTAPE1 and inhibited by Ad5/F35-siAPE1 infection (Fig. 3H).

Mutant p53 binds to DNA substrate in vivo. Western blotting showed that the cells expressed the same amount of the different p53 alleles and confirmed that the p53 mutants are defective in induction of p21 (Fig. 4A). To demonstrate the DNA binding activity of p53 *in vivo*, ChIP assays were performed on Hep3B, HepG2 and MHCC97L cells. The amount of target DNA precipitated is expressed as a percentage of the input target DNA. As shown in Fig. 4C, the p21 promoter precipitation and p21 mRNA level increased in wtp53 and mutp53 cells, compared with that in p53-null cells (Fig. 4B and C). In addition, the p21 promoter precipitation and p21 mRNA expression in mutp53 cells was much lower than that in wtp53 cells (Fig. 4B and C).

APE1 stimulates the mutp53 DNA binding to p21 promoter in vivo. As shown in Fig. 4D, a decrease in APE1, p53 and p21 protein expression was observed after infection with Ad5/F35-siAPE1, and APE1, p53 and p21 protein levels increased after the WTAPE1 infection in wtp53 cells. Ad5/F35-siAPE1 also inhibited the protein expression of APE1 and p53 in mutp53 cells, while WTAPE1 increased the APE1 and p53

protein levels (Fig. 4G). The p53 mutants were defective in induction of p21 protein in mutp53 cells (Fig. 4G).

To test whether the DNA binding activity of mutp53 is regulated by APE1 *in vivo*, ChIP assays were performed. In wtp53 cells, the WTAPE1 group showed significant increase in p21 mRNA level and p21 promoter precipitation compared with the p3XFLAG-CMV-14 empty vector, while the p21 mRNA level and p21 promoter precipitation clearly decreased in the Ad5/F35-siAPE1 treatment group compared with the Ad5/F35-EGFP group (Fig. 4E and F). p21 promoter precipitation and p21 mRNA expression were lower in mutp53 cells than in wtp53 cells. As shown in Fig. 4H, the p21 mRNA expression in mutp53 cells remained at lower levels and was not affected by APE1. Of note, WTAPE1 increased the DNA binding of mutp53 to p21 promoter, whereas silencing of APE1 inhibited the DNA binding activity of mutp53 (Fig. 4I).

Discussion

In the present study, we first examined the expression of APE1 and mutp53 in HCC tissues. Our data indicated that the increased APE1 level, cytoplasmic localization of APE1 and mutp53 expression were relevant to neoplastic alteration and poor differentiation of HCC. Notably, we observed that reduced mutp53 bound efficiently to nonlinear DNA but not to linear DNA, and APE1 could enhance DNA binding activity of mutp53 to nonlinear DNA.

Recent studies demonstrated that elevated APE1 expression was observed in several human tumors, such as ovarian cancer, cervical cancer, non-small cell lung cancer, osteosarcoma and other tumors (6,21-24). The increased APE1 expression
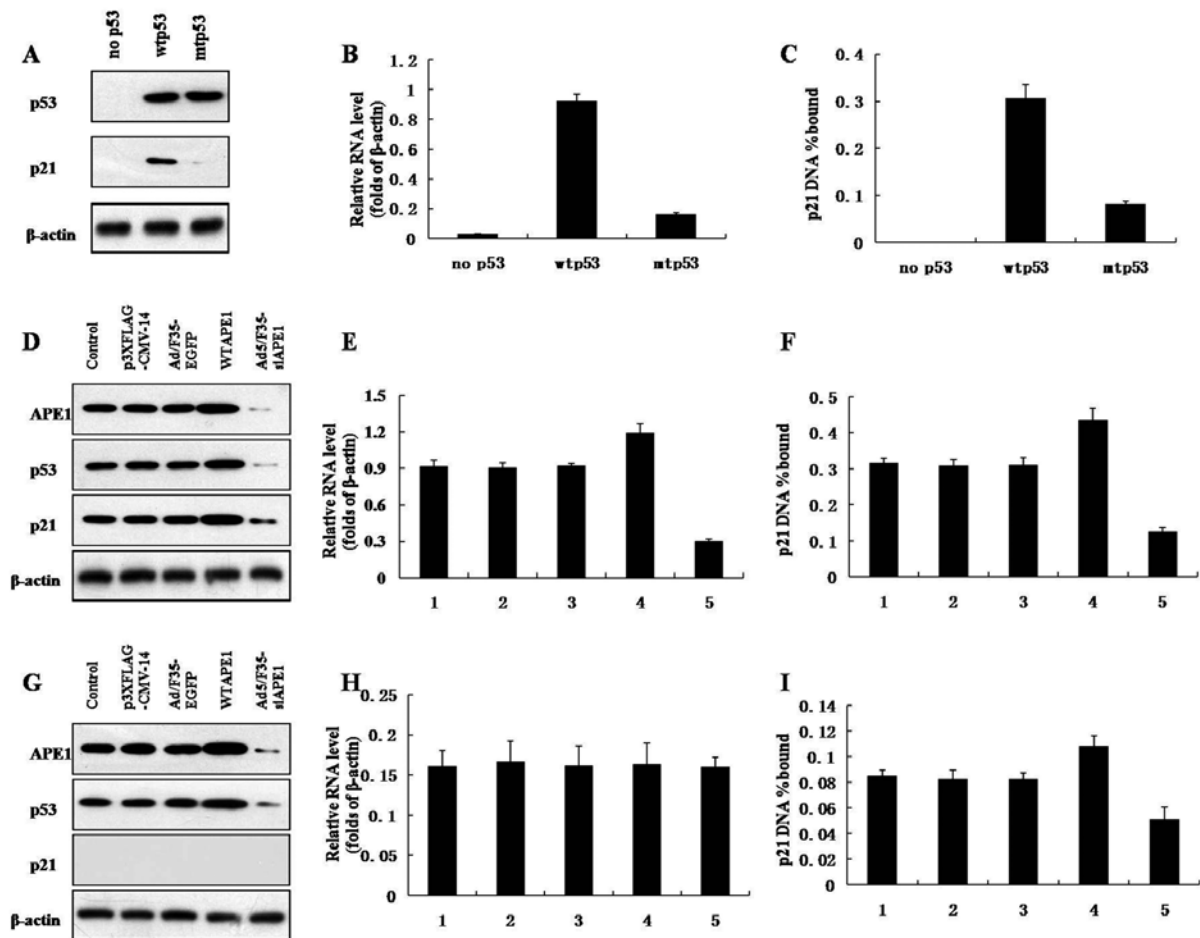
Figure 4. ChIP from hepatoma cells with different p53 status. (A) Western blotting for p53, p21 and β-actin. (B) RNA was purified from Hep3B, HepG2 and MHCC97L cell lysates and relative levels of p21 mRNA were analyzed using quantitative RT-PCR. (C) Analysis of p53 DNA binding to p21 promoter using ChIP assays. Wtp53 and mutp53 hepatoma cells were treated with WTAPE1 or Ad5/F35-siAPE1. Western blotting for APE1, p53 and p21 of HepG2 (D) or MHCC97L (G) cells. Quantitative RT-PCR for p21 mRNA level of HepG2 (E) or MHCC97L (H) cells. Quantitative PCR for ChIP of HepG2 (F) or MHCC97L (I) cells. Lane 1, control; lane 2, p3XFLAG-CMV-14; lane 3, Ad/F35-EGFP; lane 4, WTAPE1; lane 5, Ad5/F35-siAPE1.

was associated with chemo- and radioresistance, whereas downregulation of APE1 can enhance tumor sensitivity to chemo- and radiotherapy (5,6,25,26). Moreover, the tumor cells showed cytoplasmic reactivity of APE1, whereas the normal cells showed APE1 staining in the nucleus, indicating that APE1 subcellular localization has a prognostic value and correlates with aggressiveness (21,27-29). In this study, the shifts of APE1 from nucleus to cytoplasm and increased APE1 expression were correlated with neoplastic alteration and a lower degree of differentiation, which is in line with a previous study (8).

p53 is one of the most important tumor suppressor genes in the genome and encodes a transcription regulatory protein that helps preserve genomic integrity by its participation in stress-response pathways and DNA repair pathways (30,31). APE1 has been found to be a potent activator of p53 DNA binding, which can enhance p53 DNA binding by redox-dependent and -independent mechanisms (15,16,32,33). Overexpression of APE1 in tumors is associated with increased levels of p53, and they are independent predictors of prognosis and poor response to chemotherapy (34). p53 is mutated in ~50% of human cancer types including HCC (35), and its mutations not only lose their tumor suppressive activities but often gain additional oncogenic functions (13,14,36). Our data showed

that increased APE1 expression was associated with mutp53 proteins, and the combination of higher APE1 expression and mutp53 expression was correlated with a lower degree of tumor differentiation, which might be a risk factor for HCC.

In addition to the DNA repair functions (37), APE1, as a redox factor, maintains transcription factors in an active reduced state (38). In this role, APE1 stimulates the DNA binding activity of numerous transcription factors, including AP-1, NF-κB, HIF-1α, p53 and others (3,39-41). Wtp53-SSDB can occur in different modes depending on the conformation of p53-binding sites, either sequence-specific to linear DNA, or sequence- and structure-specific to nonlinear DNA. In contrast to SSDB to linear DNA, which is most probably mediated solely by the p53 core DBD (42), sequence-specific and DNA structure-dependent SSDB to nonlinear DNA and non-SSDB modes of DNA interaction involve both the DBD and the CRD (18,43-45). The complex interactions of mutp53 with DNA were shown to require both the mutp53 DBD and the intact p53 CRD (46). Although mutp53 has lost the wtp53-SSDB and could not elicit the same transcriptional response as wtp53, it requires the p53 DBD and the CRD for high-affinity binding (47,48). Gohler et al revealed that the specific and selective binding of mutp53 to nonlinear DNA provide mutp53 proteins as DNA structure-specific DNA-binding (DSSB)

proteins (17). The CRD is important for mediating stable complex formation of p53 with nonlinear DNA in mutp53-DSSB (17) and in wtp53-SSDB (18,44,45). Similar to previous studies, we found that mutp53 bound efficiently to nonlinear DNA, but not to linear DNA (17). Markedly, addition of DTT enhanced the mutp53 DNA binding, requiring a reduced p53 status as well as in wtp53 DNA binding (16).

APE1 is one of the cofactors positively influencing DNA binding of p53 *in vitro* (15) and transcription *in vivo* (33,49). As a redox factor, APE1 activates wtp53 for SSDB by reducing disulfide bonds in the p53 DBD (15,49). In addition, APE1 is also able to enhance DNA binding of reduced wtp53 by a redox-independent manner, which facilitates the formation of p53 tetramers from higher oligomeric forms as well as from dimers. Previous studies revealed that APE1 enhanced wtp53 DNA binding to both p53-RE$_{lin}$ and p53-RE$_{struct}$ (15,16). The redox-independent activation of wtp53 is due to a regulatory interaction of APE1 with the p53 CRD, as truncated p53 lacking the CRD could no longer be activated by APE1 (15). Although a stable interaction between p53 and APE1 could not be shown (15), a small fraction of the p53 and APE1 proteins (~5%) interacted in Far-Western and IP-Western assays *in vitro* (33). Furthermore, Tan *et al* showed that APE1 and p53 colocalize *in vivo* (50), supporting the theory of a specific, albeit physically weak, interaction of these proteins. The 249th codon of p53 is mutated from AGG to AGT and the amino acid from Arg to Ser (R249S) in >50% HCC patients in China, which is intact as wtp53 proteins. As CRD is essential in DNA structure-dependent binding of mutp53, which is intact in most of the frequently encountered mutp53 proteins, we investigated whether APE1 could regulate the DNA binding activity of mutp53 proteins. Our on-array binding analyses showed that APE1 enhanced mutp53 DNA binding to nonlinear DNA, but not to linear DNA. Notably, we demonstrated that APE1 also enhanced the DNA binding activity of mutp53 to p21 promoter *in vivo*. In addition, the p53 mutant is defective in induction of p21, which is in accordance with previous studies (33,49).

In conclusion, APE1 was able to stimulate DNA binding activity of mutp53, and the expression of APE1 and mutp53 was correlated with carcinogenesis and progression of HCC, which indicates that APE1 and p53 may be potential molecular therapeutic targets of HCC. The present study may contribute to a better understanding of the transcriptional regulation of p53 by APE1 and may, therefore, be the basis for the design of new clinical trials.

Acknowledgements

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