Lentiviral-mediated short hairpin RNA silencing of APE1 suppresses hepatocellular carcinoma proliferation and migration: A potential therapeutic target for hepatoma treatment

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Abstract. Apurinic/apyrimidinic endonuclease-1 (APE1) is a protein involved in DNA repair and transcriptional regulation of gene expression. APE1 expression was reported to be correlated with poor prognosis in hepatocellular carcinoma (HCC) patients. Based on our previous study, we hypothesized that APE1 may be involved in the metastatic progression of HCC. Thus, the present study aimed to investigate the knockdown effect of APE1 using shRNA in HCC and demonstrate that silencing of APE1 in MHCC97-H cells can decrease the oncogenic transforming potential in vitro and reduce the growth of HCC tumor xenografts in vivo. Silencing of APE1 expression decreased the cell proliferation and survival, reduced the cell adhesion ability in Matrigel or fibronectin-coated plates and suppressed the cell migration and invasion in a Transwell assay of HCC cells. In the xenograft study, tumor growth was markedly inhibited in the APE1-silenced group. Silencing of APE1 in MHCC97-H cells decreased the oncogenic transforming potential in vitro and reduced the growth of HCC tumor xenografts in vivo. Inhibition of APE1 may present a novel therapeutic approach for the treatment of HCC.

Introduction

Hepatocellular carcinoma (HCC) is the most common primary malignant tumor type observed in the human liver (1). HCC is also the third leading cause of cancer-related mortality worldwide, and it is especially prevalent in Southeast Asia and sub-Saharan Africa (2). Despite improvement in the treatment of HCC over the last couple of decades, the mortality rate is still high due to relapse and tumor metastasis (3,4). Increased understanding of HCC progression at the molecular level is crucial to develop novel targeted therapies which could be promising therapeutic options for HCC patients.

Apurinic/apyrimidinic endonuclease-1 (APE1) is a protein involved in DNA repair and transcriptional regulation of gene expression (5). Several studies have shown that APE1 is overexpressed in numerous types of cancer, including prostate, non-small cell lung, colorectal, ovarian and cervical cancer, and osteosarcoma (6-11). APE1 overexpression was also found to be associated with a poor prognosis and resistance to radiation and chemotherapeutic drugs (12). Since APE1 is a vital enzyme in the DNA repair pathway induced by irradiation damage, it may have a critical role in the cancer cell response to chemotherapy and radiotherapy. While the function of DNA repair has been extensively investigated, the role of activating transcription factors mediated by APE1 has not yet been widely studied (13). Previous studies indicated that APE1 stimulated the DNA binding activity of various transcription factors including activator protein-1 (AP-1), NF-κB, HIF-1α and p53 (14,15). However, the link between gene expression and related cell behavior is not well elucidated hampering the understanding of the function of APE1 in cancer cell biology.

Recently, APE1 was reported to be significantly overexpressed in HCC tissues than that in the surrounding cirrhotic tissues (16,17). In addition, we previously showed that APE1 was highly expressed in a poorly differentiated group, capsular invasion group and metastasis group (18). We also found that
APE1 protein is mainly expressed in the nuclei in normal liver tissues. While in malignant liver tissues, APE1 protein is expressed in the nucleus and cytoplasm. Therefore, we hypothesized that APE1 may be involved in the metastatic progression of HCC. Based on previous results (18), this study aimed to investigate the knockdown effect of APE1 using shRNA in HCC and demonstrate that silencing of APE1 in MHCC97-H cells can decrease the oncogenic transforming potential in vitro and reduce the growth of HCC tumor xenografts in vivo. It was demonstrated that inhibition of APE1 may present a novel therapeutic approach for the treatment of HCC.

Materials and methods

Cell line. The MHCC97-H cell line was obtained from the Liver Cancer Research Institute of Fudan University (Shanghai, China). The cells were cultured at 37°C in a humidified incubator under 5% CO2 and grown in Dulbecco's modified Eagle's medium (DMEM; Hyclone Company, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; PAA Laboratories GmbH, Pasching, Austria), 100 u/ml penicillin, and 100 µg/ml streptomycin.

Lentiviral vectors and infection. The APE1 RNA interference lentivirus vector named LV-APE1-shRNA and LV-NC-shRNA (control) were constructed at GeneChem Technology (Shanghai, China) as previously described (19). The shRNA targeting sequencing for APE1 was: 5'-GAGACCAATGCTGAGGTAC-3'. The MHCC97-H cell line was infected with LV-APE1-shRNA or LV-NC-shRNA for 2 h and subsequently placed in fresh medium, while the uninfected cells were used as the blank control. The cells were cultured for the next 48 h and then harvested for western blot analysis or prepared for the following experiments.

Western blotting and RT-PCR. Western blot analysis was performed as previously described (19) using the antibodies as follows: mouse anti-APE1 (Novus Biologicals, Littleton, CO, USA), anti-c-Fos polyclonal, anti-c-Jun, anti-caspase-3 and anti-inducible matrix metalloproteinase (MMP)-2 and a monoclonal antibody against β-actin (Merck KGaA, Darmstadt, Germany). The protein concentration was analyzed as APE1/β-actin. Image J software (National Institutes of Health, Bethesda, MD, USA) was used for densitometric analysis.

Table I. Detected genes and primer sequences.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tbody>
<tr>
<td>APE1</td>
<td>5'-AGAGCCAGGCCCAAGAGAAGATG-3'</td>
<td>5'-GAAGCCATTCCACTTCAAAGAG-3'</td>
</tr>
<tr>
<td>c-Fos</td>
<td>5'-GAGAACAGCCAGACTGGCCCG-3'</td>
<td>5'-GTTGAACCCAGAGACATC-3'</td>
</tr>
<tr>
<td>c-Jun</td>
<td>5'-TCAGACAGTGCCCCGAGATG-3'</td>
<td>5'-CTGCGTTAGCATGAGTTGG-3'</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>5'-AGCTGGTTCCATGAAGCCAG-3'</td>
<td>5'-CGTGGACCATCATCCACAATAC-3'</td>
</tr>
<tr>
<td>MMP-2</td>
<td>5'-GTGCCCAGAAATAGTGTGCAGC-3'</td>
<td>5'-CGTGGAGGACTGTAAGTGATG-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-CCTGCACCACCAACTGCTTCA-3'</td>
<td>5'-ACCACCTGACAGTTGGCAG-3'</td>
</tr>
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</table>

Cell proliferation. Cell proliferation of the infected and control cells was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma, New York, NY, USA). Briefly, the cells were seeded at 3,000 cells/well and were cultured for 48 h prior to measurement. The cells were then washed with phosphate-buffered saline (PBS; Biovision, San Francisco Bay, CA, USA) and then incubated with 0.45 mg/ml MTT solution made up in serum-free media at 37°C. Once the formation of blue formazan crystals was apparent, the MTT solution was removed. Subsequently, 100 µl of DMSO (Sigma) was added to solubilize the blue formazan crystal, and the absorbance was read at 570 nm.

Cell adhesion. The adhesion assay was performed as previously described (20). Briefly, a 96-well plate was pre-coated with Matrigel and fibronectin (FN) (both from BD Biosciences, New York, NY, USA), dissolved in 50 µl of PBS, incubated for 2 h at 37°C and then blocked for non-specific sites by the addition of 1% bovine serum albumin (BSA) in 100 µl of PBS/well for 30 min. Cells with 90% confluency were harvested with a brief treatment of 0.25% trypsin (Invitrogen). The cells were plated in 96-well plates at 1x10^3 density and cultured for 1 h. The medium was aspirated and the cells were washed twice with PBS to remove unattached cells. The viability of the attached cells was determined by MTT assay. All experiments were performed in triplicate with 4–8 replicates/experiment.

Cell migration and invasion. Cell migration and invasion assays were performed in BioCoat Transwell chambers (Corning Costar, Tewksbury, MA, USA). While cell migration was determined with uncoated porous inserts, cell invasion was measured using a filter precoated with Matrigel. Cells were starved under a serum-free condition 24 h prior to experimentation. Subsequently, the cells were plated on the upper chamber with 0.2% PBS and cultured for 24 h. Migrated and invaded cells on the inserts were fixed using 90% ethanol following the manufacturer's instructions. Subsequently, reverse transcription was performed using the PrimerScript RT reagent kit (Takara Biotechnology, Dalian, China). Primers for APE1, c-Fos, c-Jun, caspase-3 and MMP-2 were designed as listed in Table I. PCR reaction was performed with 30 cycles (Piko TCP9600; Thermo Scientific, Waltham, MA, USA). The gene expression levels were analyzed as APE1/GAPDH. Densitometric analysis was performed using Image J software.
followed by staining with 0.1% crystal violet (Sigma) for counting. The numbers of migrated and invaded cells were counted over three fields per one filter for triplicate experiments.

**Xenograft tumor growth.** BALB/c nude mice (Shanghai Slaccas Co., Shanghai, China) (4-5 weeks old) were used in the xenograft growth study. The animal study was performed according to procedures approved by the Animal Care and Ethics Committee of Fujian Medical University. Tumor growth was measured as follows. Briefly, a volume of 0.2 ml of 2x10^6 viable LV-APE1-shRNA or LV-NC-shRNA cells was subsequently injected into the right axilla of BALB/c nude mice (n=6 per group). Tumor volumes were analyzed every two days by measuring the major axis (a) and the minor axis (b) by Vernier calipers. The total volume (V) was calculated by the equation: V = 1/6 π a b^2. After 3 weeks, the nude mice were euthanized, and the xenografts were resected for weight measurement. The inhibitory rate of tumor growth was calculated using the following equation: Inhibitory rate = 1 - (average weight of experimental group - average weight of control group) x 100%. Subsequently, tissue samples were collected for histological and RT-PCR analyses.

**Histological analysis.** Histological analysis was performed as described previously (21). In brief, 5-µm sections were stained with hemotoxylin and 1% eosin Y solution separately. After that, the sections were rehydrated in a graded series of ethanol solutions, and then the ethanol was extracted with xylene. Mounting medium was added and the slide was covered with a coverslip prior to observation using a microscope. Apoptosis was measured using the terminal-deoxynucleotidyl transferase deUTP nick end labeling (TUNEL) method carried out using the TUNEL apoptosis assay kit (C1091; Beyotime, Shanghai, China) according to the manufacturer's instructions. Briefly, the sections were incubated with 0.3% H2O2 for 30 min. After washing with PBS, the sections were treated with biotin-16-deUTP in reaction buffer at 37˚C for 60 min. Subsequently, the specimens were incubated with streptavidin-peroxidase complex and stained with DAB after twice with PBS (both from Maixin, Fuzhou, China). Finally, cell morphology was observed under a light microscope (Olympus BX43; Olympus Co., Tokyo, Japan).

**Statistics.** Data are expressed as mean ± SEM from a representative experiment conducted in triplicate. Results were analyzed using unpaired two-tailed Student's t-test and P-values <0.05 were applied to determine statistically significance by comparing data sets.

**Results**

**shRNA silencing of APE1 expression effectively reduces APE1 mRNA and protein expression in the MHCC97-H cells.** MHCC97-H is a highly metastatic HCC cell line, which expresses high endogenous APE1 according to our previous results. In this study, MHCC97-H cells were infected by a lentivirus which contained APE1 shRNA (LV-APE1-shRNA) or an empty vector (LV-NC-shRNA). Following infection, the cells were collected for mRNA and protein expression analysis by RT-PCR and western blotting. Uninfected MHCC97-H cells were included as the negative control. Compared with the LV-NC-shRNA cells and uninfected negative control cells, the mRNA and protein expression levels of APE1 in the LV-APE1-shRNA cells was decreased. Based on the RT-PCR results, the mRNA level of APE1 was reduced by 90.6% in the LV-APE1-shRNA cells, while the protein level of APE1 was significantly reduced by 72.4% in the LV-APE1-shRNA cells vs. that in the LV-NC-shRNA and control cells as detected by western blotting (Fig. 1A and B).

**APE1 silencing significantly reduces MHCC97-H cell proliferation.** After infection, MHCC97-H cell proliferation rates were evaluated in the LV-APE1-shRNA, LV-NC-shRNA and negative control cells. As shown in Fig. 1C, cell proliferation was markedly decreased by knockdown of APE1 in the LV-APE1-shRNA cells (0.2871±0.1118) compared to the LV-NC-shRNA (0.5213±0.2551) and negative control (0.5515±0.2659) cells. However, the proliferation rate was almost equal in the LV-NC-shRNA and blank control groups.
APE1 silencing inhibits HCC cell adhesion ability. Cell adhesion was evaluated by cell-matrix adhesion, which was measured by MTT assay to reflect the cell adhesion ability. As shown in Fig. 2A, cell adhesion was significantly inhibited in the LV-APE1-shRNA cells compared to that in the LV-NC-shRNA and negative control cells in the Matrigel-coated plates. The data were similar with the results using FN-coated plates (Fig. 2B). Thus, silencing of APE1 significantly inhibited HCC cell adhesion ability.

APE1 silencing reduces cell invasive ability. Cell migration and invasion were investigated by Transwell assays. Cell migration was slightly decreased following the silencing of APE1 in vitro. However, cell invasion was markedly reduced in the LV-APE1-shRNA cells compared to that in the LV-NC-shRNA and negative control cells (Fig. 2C). Cell counting results showed that the cell migration in the LV-APE1-shRNA cells was decreased ~26% compared to the LV-NC-shRNA and negative control cells (Fig. 2D).

APE1 knockdown inhibits HCC tumor xenograft growth. APE1-knockdown MHCC97-H cells were subcutaneously injected into nude mice to investigate tumor growth in vivo. Consistent with the in vitro data presented above indicating that proliferation was significantly enhanced in cells infected with LV-NC-shRNA and markedly reduced in APE1-depleted cells, tumor growth was observed in the nude mice injected with LV-NC-shRNA MHCC97-H cells and compared to those injected with LV-APE1-shRNA MHCC97-H cells. The volume of tumors was also measured. Firstly, the volume of the tumor xenografts from the LV-APE1-shRNA group showed a marked reduction in growth rate compared to tumors obtained from the LV-NC-shRNA and negative control groups after 9 days. Furthermore, the final weight of the tumor xenografts from the LV-APE1-shRNA group was ~315% less than the final weight of the tumor xenografts from the LV-NC-shRNA and negative control groups (Fig. 3). The inhibition rate was 75.9% in the LV-APE1-shRNA group compared to the control groups in the final stage by measuring the weight of the xenografts (Table II).
Gene and protein expression profile analysis. RNA and protein were extracted from the xenografts, and expression levels of various genes were assessed by RT-PCR and western blotting. Compared with negative control, the LV-APE1-shRNA group showed a significantly decrease in mRNA expression of c-Fos (47.580%), c-Jun (47.402%), caspase-3 (65.355%) and MMP-2 (64.369%) (Fig. 4A). These observations were consistent at the protein level, where the LV-APE1-shRNA group exhibited a significant decrease in c-Fos, c-Jun, caspase-3 and MMP-2 expression by 71.872, 80.399, 75.616 and 88.393%, respectively (Fig. 4B).

APE1 silencing accelerates the apoptosis of HCC cells and reduces the number of irregular mitoses. When comparing the different samples from the LV-APE1-shRNA, LV-NC-shRNA and negative control groups, we identified a positive qualitative difference caused by silencing of APE1. From the H&E staining results, less tissue necrosis and more small cancer cell apoptotic bodies (indicating as red dye and small round body) were observed in the LV-APE1-shRNA tumors compared to the LV-NC-shRNA and negative control tumors (Fig. 5A). Furthermore, according to the pathological classification, the irregular mitosis was grouped into different types, such as tri-pole, asymmetrical and popcorn-like. By counting the number of irregular mitoses, we found that irregular mitotic tumor cells numbered <5/HPF in the LV-APE1-shRNA group, while these numbers in the LV-NC-shRNA and negative control groups were >5/HPF.

Apoptosis index can be calculated by counting the apoptotic cell number using the TUNEL method. As shown in Fig. 5B, a higher number of apoptotic cells was observed as black dots in the LV-APE1-shRNA group when compared to the LV-NC-shRNA and negative control groups in the TUNEL-stained tissue samples. The apoptosis index was significantly increased in the LV-APE1-shRNA group which was almost double than that in the LV-NC-shRNA and negative control groups (Fig. 5C).

Table II. Silencing of APE1 inhibits tumor formation of the MHCC97-H cell-derived tumors in vivo.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Weight of tumors (g)</th>
<th>Inhibition ratio (%)</th>
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<tbody>
<tr>
<td>LV-APE1-shRNA</td>
<td>6</td>
<td>0.267±0.082</td>
<td>73.772</td>
</tr>
<tr>
<td>LV-NC-shRNA</td>
<td>6</td>
<td>1.018±0.160</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>1.107±0.178</td>
<td>-</td>
</tr>
</tbody>
</table>

*P<0.05

Gene and protein expression profile analysis. RNA and protein were extracted from the xenografts, and expression levels of various genes were assessed by RT-PCR and western blotting. Compared with negative control, the LV-APE1-shRNA group showed a significantly decrease in mRNA expression of c-Fos (47.580%), c-Jun (47.402%), caspase-3 (65.355%) and MMP-2 (64.369%) (Fig. 4A). These observations were consistent at the protein level, where the LV-APE1-shRNA group exhibited a significant decrease in c-Fos, c-Jun, caspase-3 and MMP-2 expression by 71.872, 80.399, 75.616 and 88.393%, respectively (Fig. 4B).

Figure 3. Silencing of APE1 inhibits tumor formation in the MHCC97-H cell-derived tumors in vivo. (A) Tumors in the different nude mouse groups injected with LV-APE1-shRNA, LV-NC-shRNA or control cells. (B) Measurement of tumor volume after inoculation from day 6 to 21. "P<0.01,""P<0.001.

Figure 4. Expression analysis of various genes at the mRNA and protein levels. (A) mRNA expression analysis by RT-PCR. (B) Protein expression analysis by western blotting.
The poor prognosis of HCC patients is mainly due to the high frequency of late-stage cancer and metastasis at diagnosis. Despite benefits from surgical resection and a low risk of complications, only a limited proportion of HCC patients are eligible to undergo optional resection at diagnosis. Chemotherapy represents the main therapeutic option for these HCC patients, but neither single drug nor multiple drug treatments prolong the survival of late-stage HCC patients (22). In order to improve therapeutic efficacy, targeted drugs such as sorafenib are currently used in late-stage HCC patient. Sorafenib is a multikinase inhibitor that has already been approved for the treatment of advanced HCC (23). Combination of chemotherapy drugs with a targeted drug offers a novel therapeutic approach for HCC treatment. However, the adverse effects and acquisition of drug resistance of patients to sorafenib remain a problem in the clinic (24). It is necessary to explore more promising and specific targeted drugs for HCC particularly for late-stage HCC patients with metastasis.

APE1 is a multifunctional gene which regulates DNA repair and related gene expression during cell damage and cancer development. Based on our previous study, APE1 was shown to be highly expressed in poorly differentiated, capsular invasion and metastasis groups (18). In addition, we also found that APE1 protein is mainly expressed in the nuclei in normal liver tissues, while in malignant liver tissues, APE1 protein is expressed in the nucleus and cytoplasm. Such a differentiated expression pattern of APE1 indicates that it can be a potential marker for cancer diagnosis and treatment. Previously, the function of APE1 in HCC was found to be associated with cell apoptosis and resistance to radiotherapy and chemotherapy (10). Cun et al found that knockdown of APE1 increased the sensitivity of human HCC cells to radiotherapy in vitro and in vivo (12) and Zou et al demonstrated that inhibition of APE1 by a small-molecule inhibitor suppresses pancreatic cancer cell proliferation and migration (25). Moreover, inhibition of APE1 by small-molecular agents also inhibited the growth of tumor endothelium and endothelial progenitor cells (26). These results suggested an important role of APE1 in cancer and that the function of APE1 can be investigated by silencing of APE1. Lentiviral-mediated silencing of genes through RNA interference is an ideal approach that can achieve long-lasting transgene expression (27). In the present study, we selected lentiviral-mediated shRNA silencing of the APE1 gene, which can provide more stable expression compared to atopic transfection using liposome transfection.

Previous studies have shown that APE1 is a redox activator in the cell nucleus, which can activate the DNA binding activity of transcription factors associated with cancer development such as AP-1, NF-κB and P53 (28,29). The AP-1 complex has been implicated in the transformation and progression of cancer (30). It was reported that activation of AP-1 transcription factors is frequently found in the early event of human HCC development (31). Firstly, AP-1 is comprised of heterodimers of Fos and Jun family proteins, which bind to a consensus DNA sequence that are often located in the target promoter region of genes. c-fos and c-jun are proto-genes that are important in cell cycle progression as well as proliferation (32). Secondly, altering the expression of AP-1 component proteins also affects cellular invasion (33). AP-1-regulated genes play a role in the invasive process. For example, inducible matrix metalloproteinases (MMPs) share a consensus AP-1 binding site in their promoter, which is found to be expressed in various invasive tumors (34). In addition, Fishel et al found that c-Fos can induce expression of MMP-1, MMP-2 and MMP-3 and initiate tumor progression (35). On the contrary, invasiveness of cells was significantly reduced when expression of the AP-1 component and AP-1 protein activity were inhibited (32). In the present study, we found that silencing of APE1 decreased c-Fos and MMP-2 expression, which consequently inhibited cell-matrix adhesion, cell migration and invasion. These results indicate that AP-1 is one of the downstream effectors of APE1, confirming APE1 as regulator of cancer cell proliferation and migration.

Targeted APE1 therapy was assessed in a previous study, suggesting that APE1 expression level and its deregulation in
cells can be used as a therapeutic target for tumor radiotherapy and chemotherapy sensitivity prediction (35). Our data validated the possibility of using APE1 as a target for inhibiting tumor growth in vivo. In the LV-APE1-shRNA group, the final weight of the tumor xenografts was ~315% less than that in the control group. The inhibition rate was 75.9% in the LV-APE1-shRNA group compared to the control groups in the final stage by measuring the weight of xenografts. This result is in line with previous results which found that inhibition of APE1 reduced the expression of c-Fos, resulting in a decrease in cell migration and invasion (28). Moreover, silencing of APE1 enhanced the sensitivity of HCC cells to radiotherapy (12). Therefore, APE1 as a tumor suppressor in HCC could be used in targeted therapy combined with other treatment strategies such as surgery, chemotherapy, radiotherapy and immunotherapy.

In conclusion, the present study demonstrated that APE1 silencing by shRNA suppressed the proliferation and mobility of HCC cells. Firstly, silencing of APE1 expression in HCC cells decreased cell viability and survival compared with the control cells in vitro while the cell proliferation rate in the APE1-silenced group was significantly higher than that in the control cells. In addition, APE1 was important in cell-matrix adhesion, cell migration and invasion. Silencing of APE1 expression markedly decreased cell adhesion in Matrigel or FN-coated plates. Moreover, cell migration and invasion were also decreased when APE1 expression was depleted. Finally, xenograft growth was inhibited by silencing of APE1 expression compared with that in the control groups in nude mice. Activation of protein expression levels were found to be associated with APE1 expression by western blot and RT-PCR analyses. Expression of c-Fos and c-Jun were downregulated by silencing of APE1, while expression of caspase-3 and MMP-2 were also decreased. These results suggest that APE1 is an important gene in the regulation of HCC cell proliferation and mobility by regulating related gene expression. These findings suggest that APE1 may be useful in cancer targeted drug development for inhibition of cancer metastasis.

Acknowledgements

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References


