Role of DNA damage in the progress of chronic tubule-interstitial injury

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Abstract. Tubulointerstitial fibrosis (TIF) is a common final endpoint of chronic allograft nephropathy. Over the years, several hypotheses have been developed to explain the progression of TIF, including mechanisms such as inflammation, epithelial-mesenchymal transition, senescence, chronic hypoxia and reactive oxygen species. Furthermore, TIF is reportedly induced by the 'damage-proliferation-death' cycle. In the present study, an AA renal fibrosis model was established *in vitro* to investigate whether the vicious proliferation-death cycle is a pathophysiological process of TIF following chronic injury to the kidneys. Results from the present study revealed that cell death was associated with the entrance of cells into the cell cycle. Genetic knockdown of p21 was observed to increase cell cycle progression and the proliferative rate of cells, which overall promoted increased rates of cell death. In addition, the activation of the DNA damage response (DDR) signaling pathway was demonstrated to be crucial to the initiation of the vicious cycle of 'proliferation-death'. Ataxia telangiectasia mutated (ATM) is an important molecule of the DDR and the genetic knockdown of ATM induced apoptosis, increased cell proliferation and promoted cell death. The increase in apoptosis was suggested to be due to the decreased expression levels of p21 observed following the genetic knockdown of ATM. In conclusion, the present study suggested that the crosstalk between the ATM and p21 protein may serve an important role in the regulation of the 'proliferation-death' cycle in the progress of chronic tubulointerstitial injury.

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Abbreviations: AA, aristolochic acid; ATM, ataxia telangiectasia mutated; Chk2, checkpoint kinase 2; DDR, DNA damage response; HK-2, human kidney 2; PCNA, proliferating cell nuclear antigen; r-H2AX, H2AX phosphorylation at Serine 139; TIF, tubulointerstitial fibrosis

Key words: cell proliferation, cell death, DNA damage response, p21, tubulointerstitial fibrosis

Introduction

Chronic kidney disease (CKD) is an increasing public health problem with substantial health care costs and morbidity (1). Tubulointerstitial fibrosis (TIF) is a common result of chronic kidney disease; it leads to destruction of the normal kidney structures and irreversible loss of kidney function (2,3). The induction of TIF causes the disease to progress to renal failure (4), and several hypotheses have been developed to elucidate the progression of TIF, including mechanisms such as inflammation, epithelial-mesenchymal transition, senescence, chronic hypoxia and the contribution of reactive oxygen species (5,6). The progression of TIF has been found to be strongly correlated with renal tubular lesions (7). Renal tubular epithelial cells (RTECs) are the most abundant cell type in the kidney and produce various active factors, such as growth factors, interleukins, inflammatory factors, chemokines and cell adhesion molecules (8,9). Thus, RTECs are involved in numerous processes, such as epithelial cell transdifferentiation, inflammatory cell activation and cell proliferation, and notably, they have also been observed to serve an important role in TIF progression (9,10).

RTECs were originally considered as terminally differentiated cells; however, they have since been found to exert significant proliferative ability (11). Kidney injury has been discovered to stimulate RTECs to release cytokines and rapidly enter the cell cycle, which has been demonstrated to be important for the rapid recovery of renal function following acute injury (12,13). Proliferation is accompanied by the presence of damage factors (12); RTECs are highly sensitive to damage factors upon entering the cell cycle, and when the damage factors persist, they are also found to have an adverse effect on the subsequent proliferation response (14-16). In a TIF model, RTEC injury was found to be positively correlated

with compensatory proliferation, in which the proliferation of RTECs subsequently promoted cell death (17,18). The sensitivity of cells to kidney injury is different, that is, differentiated complete cells are more sensitive compared with stem cells, which are more sensitive compared with proliferating cells (14,19). Upon injury, the stimulation of surviving RTECs to enter the division cycle has been found to increase their sensitivity to the external environment, such as hypoxia and drug toxicity, which promotes the death of RTECs; therefore, a vicious circle of 'proliferation and death' is formed (10). In this cycle, the signaling pathways in RTECs are contradictory; however, the specific mechanisms involved remain unclear.

The DNA damage response (DDR) is a multicomplex network of signaling pathways that are involved in DNA damage repair, cell cycle checkpoints and apoptosis (20). A previous tumor cell study showed that drugs or radiation will not cause cell death due to damage to DNA, which may be associated with proliferative and death-associated mechanisms (21). Ataxia telangiectasia mutated (ATM) serves a central role in phosphorylating several important proteins that activate the DDR and mobilize this intricate DDR network (22).

Aristolochic acids (AA) are nephrotoxic and carcinogenic phytochemicals found in many plant species (23). AA-dependent human nephropathy occurs as the result of the environmental exposure to *Aristolochia* subspecies or its use as a traditional botanical therapy and is characterized by severe renal fibrosis and upper urothelial carcinoma (24). In the present study, an AA renal fibrosis model was established *in vitro* to investigate whether the vicious proliferation-death cycle is a pathophysiological process of TIF following chronic injury to the kidneys. In addition, the underlying molecular mechanisms of the proliferation-death cycle were investigated in the TIF model. It was hypothesized that this malignant cycle of RTECs serves as the main driver of TIF progression following the persistent release of injury factors, and that DDR-induced cell death serves an important role in its molecular mechanism.

Materials and methods

Reagents. FBS, trypsin and RPMI-1640 medium were obtained from Gibco (Thermo Fisher Scientific, Inc.), and streptomycin and penicillin were purchased from Sigma-Aldrich (Merck KGaA). AA was obtained from Chengdu Manst Biotech Co., Ltd. (http://www.cdmust.com/).

Cell culture and transfection. The human proximal tubular epithelial cell line, human kidney (HK)-2, was purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. Cells were cultured in RPMI-1640 medium, supplemented with 10% FBS and 1% antibiotics (0.1 mg/ml streptomycin and 100 U/ml penicillin), and maintained in a humidified with 5% CO_2 at 37˚C.

Cells were subsequently transfected with lentiviral vectors containing short hairpin (sh)RNA (Gima Gene) targeting p21 (shp21), ATM (shATM) or shRNA-negative control (shCon). The lentiviral vector system consists of three plasmids: GV lentiviral vector series, pHelper 1.0 vector and pHelper 2.0 vector. http://www.genechem.com.cn/Zaiti.aspx?zt=GV115. Briefly, HK-2 cells were plated and cultured for 12 h at 37˚C and subsequently, lentiviral vectors encoding shp21, shATM or shCon (45 μg/ml) were mixed with the culture medium. Polybrene, at a final concentration of 8 μg/ml, was added to the culture medium to facilitate the transfection. Following incubation for 6 h at 37˚C, fresh RPMI-1640 medium was added to the cells and cultured for a further 48 h at 37˚C. When cells reached 70-90% confluence, the medium of the transfected cells was replaced with fresh RPMI-1640 medium containing 10% FBS and 20 μg/ml AA, whereas the control groups (untreated group) were replaced with normal RPMI-1640 medium containing 10% FBS. The cells were then cultured for 24 h before the cells were collected for subsequent analysis.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and total RNA was reverse transcribed into cDNA (Takara Bio, Inc.) according to the manufacturers' protocol. RT-qPCR was subsequently performed using the SYBR® Green Master Mix kit (Takara Bio, Inc.) and a CFX96 Touch Real-Time PCR Detection system (Bio-Rad Laboratories, Inc.) according to the manufacturers' protocol (holding Stage, 95˚C for 3 min; cycling stage, 95˚C for 5 sec, 60˚C for 30 sec, 40 cycles; melt curve stage, 95˚C for 15 sec then 60˚C for 1 min). The following primer pairs were used for the qPCR: p21, forward 5'-TCTCAGGGTCGAAAACGG-3', reverse 5'-TGGGCGGATTAGGGCTTC-3'; ATM, forward 5'-ATAGATTGTGTAGGTTCCGATGG-3', reverse 5'-CATCTTGTCTCAGGTCATCACG-3'; GAPDH, forward 5'-GTGAACCATGAGAAGTATGACAAC-3', reverse 5'-CATGAGTCCTTCCACGATACC-3'. Expression levels normalized to the internal reference gene GAPDH and were quantified using the $2^{-\Delta\Delta Cq}$ method (25).

Western blotting. The cells were homogenized in ice-cold RIPA lysate (Beyotime Institute of Biotechnology) for cleavage, and phosphatase inhibitor (Beyotime Institute of Biotechnology) and PMSF (Swiss Roche, Inc.) were added at the same time (RIPA lysate:phosphatase inhibitor:PMSF=100:10:1). Western blot analysis was performed as previously described (26). Total protein was extracted from transfected HK-2 cells and quantified using a BCA assay kit (Beyotime Institute of Biotechnology). A total of 60 μg of protein were separated by 10% SDS-PAGE and subsequently transferred onto polyvinylidene fluoride membranes (0.2/0.45 μm; EMD Millipore) at 300 mA and blocked with 5% nonfat milk for 1 h at room temperature. The membranes were incubated at 4˚C overnight with primary antibodies against the following target proteins: Phosphorylated (p)-ATM (rabbit; 1:1,000; cat. no. ab81292; Abcam); ATM (mouse; 1:2,000; cat. no. ab78; Abcam); p-checkpoint kinase-2 (p-Chk2; rabbit; 1:1,000; cat. no. 64o0492; Affinity Biosciences); checkpoint kinase 2 (Chk2; rabbit; 1:5,000; cat. no. ab109413; Abcam); rabbit histone H2 A.X (r-H2AX (rabbit; 1:1,000; cat. no. ab11175; Abcam); p-p53 (rabbit; 1:1,000; cat. no. ab1431; Abcam); p53 (mouse; 1:1,000; cat. no. ab26; Abcam) p21 (rabbit; 1:1,000; cat. no. BS6561; Bioworld Technology, Inc.); CDK2 (rabbit; 1:1,000; cat. no. ab32147; Abcam); cyclin D1 (rabbit; 1:1,000; cat. no. ab16663; Abcam); proliferating cell nuclear antigen (PCNA; mouse; 1:1,000; ab29; Abcam); Bax (rabbit; 1:1,000; cat. no. 2772S; Cell Signaling Technology, Inc.); Bcl-2 (mouse;

1:1,000; cat. no. 15071; Cell Signaling Technology, Inc.) and anti-GAPDH (rabbit; 1:10,000; cat. no. AP0063; Bioworld Technology, Inc.). Following the primary antibody incubation, the membranes were washed with TBST (0.1% Tween-20) and subsequently incubated with a horseradish peroxidase-conjugated goat anti-rabbit (rabbit; 1:5,000; cat. no. BL003A; BioSharp Technology, Inc.) or anti-mouse IgG secondary antibody (mouse; 1:5,000; cat. no. BL001A; BioSharp Technology, Inc.) for 1 h at room temperature. Protein expression levels were normalized to GAPDH. Protein bands were visualized using an enhanced chemiluminescence kit and protein expressions were semi-quantified using Image-Pro Plus software 6.0 (Media Cybernetics, Inc.).

Flow cytometric analysis of apoptosis. Transfected HK-2 cells were harvested, routinely digested with trypsin and washed twice with PBS. The cells were subsequently resuspended in 500 μl binding buffer and stained with 5 μl Annexin V-FITC and 5 μl propidium iodide for 10 min at room temperature in the dark (Apoptosis Detection kit, C1062M, Beyotime Institute of Biotechnology). Apoptotic cells were subsequently analyzed using a CytoFLEX flow cytometer (Beckman Coulter, Inc.), according to the manufacturer's protocol. The results were analyzed with FlowJo 7.6 software (FlowJo LLC).

Caspase-3 activity measurement. Caspase-3 activity was analyzed using a Caspase-3 Activity Assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. Briefly, attached and floating cells were lysed for 15 min at 4˚C after centrifugation at 4˚C ($2x10^6$ cells added to 100 μl lysate) and then incubated with Ac-DEVD-p-nitroaniline (p-NA) for 1 h at 37˚C. The levels of p-NA, which reflects the caspase-3 activity, were then determined at a wavelength of 405 nm with a microplate reader.

Statistical analysis. Statistical analysis was performed using SPSS 20.0 software (IBM Corp.) and data were presented as the mean ± standard error of the mean. Statistical differences were determined using a one-way ANOVA, followed by Tukey's post hoc test for multiple comparisons, whereas an ANOVA and Tamhane T2 post hoc test was performed with data demonstrating a heterogeneous variance. All data were obtained from >3 independent experiments. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Successful transfection of lentiviral-vectors encoding shRNAs into HK-2 cells. To confirm that the shRNA vectors have been successfully transfected into the HK-2 cells, the mRNA expression levels of p21 and ATM following transfection were analyzed using RT-qPCR. shp21 and shATM were successfully transfected into HK-2 cells, as demonstrated by the significantly reduced expression levels of p21 and ATM, respectively, compared with the shCon groups (Fig. 1A and B).

p21 knockdown promotes cell cycle progression and cell proliferation in HK-2 cells. To investigate the effects of p21 on the cell cycle and cell proliferation, the protein expression levels of CDK2, cyclin D1 and PCNA following AA-induced injury were analyzed using western blotting (Fig. 2A and B). The shCon + AA group demonstrated significantly increased p21 protein expression levels and significantly decreased CDK2 and cyclin D1 protein expression levels compared with non-AA treated shCon group; however, the shp21 + AA group significantly inhibited the expression levels of p21 and significantly increased the expression levels of CDK2 and cyclin D1 proteins compared with the shCon + Aa group. In addition, following AA-induced injury, cell proliferation was increased, as demonstrated by increased PCNA expression levels, whereas following p21 knockdown with shRNA (shp21 + AA group), PCNA expression levels were significantly increased and cell proliferation was promoted compared with shCon + AA.

p21 knockdown increases AA-induced apoptosis in HK-2 cells. The apoptotic role of p21 in AA-treated HK-2 cells was subsequently investigated; AA treatment significantly increased the protein expression levels of Bax and decreased the protein expression levels of Bcl-2 in the shCon + AA group compared with the non-AA treated shCon group, whereas p21 knockdown (shCon or shCon + AA) significantly increased the protein expression levels (Fig. 3A-C). The protein ratio of Bcl-2/Bax also increased or decreased correspondingly (Fig. 3D). In addition, AA-induced injury significantly increased the apoptotic rate in the shCon + AA group compared with the shCon group (Fig. 3E and F). Under the same conditions (shCon or shCon + AA), shp21 transfection significantly increased the number of AA-induced apoptotic cells compared with the non-shp21 transfection group (Fig. 3E and F). Caspase-3 activity was also analyzed using a caspase-3 activity kit; it was observed that p21 knockdown significantly increased caspase-3 activity in the AA-induced HK-2 cells compared with the shCon + AA group (Fig. 3G). These results suggested that p21 deficiency may accelerate AA-induced RTEC apoptosis.

p21 knockdown decreases AA-induced DDR activity in HK-2 cells. The ability to induce cell death during the cell cycle through the DDR signaling pathway was investigated using a p21 gene knockdown cell model. The protein expression levels of p-ATM, ATM, p-Chk2, Chk2, rH2AX, p-p53 and p53 were analyzed using western blotting (Fig. 4). In AA-treated cells following p21 knockdown, the expression levels of these phosphorylated proteins were decreased compared with the shCon + AA group. Shp21 treatment significantly decreased the protein ratio of p-p53/p53, p-ATM/ATM and p-Chk2/Chk2 in the shCon or shCon + AA group compared with the non-p21 knockdown group (Fig. 3C-E). These finding indicated that p21 may exert a protective effect over cell survival and can increase the DNA repair ability of cells.

Knockdown of ATM expression levels using shRNA reduces the DDR and decreases p21 expression to induce cell cycle arrest. The protein expression levels of p-ATM, ATM, p-Chk2, Chk2, r-H2AX, p-p53, p53, p21, CDK2, and cyclin D1 in shCon- or shATM-transfected cells following 24 h of AA treatment were analyzed using western blotting (Figs. 5 and 6). Cells in the shATM + AA group demonstrated a decreased DNA repair ability through the observed

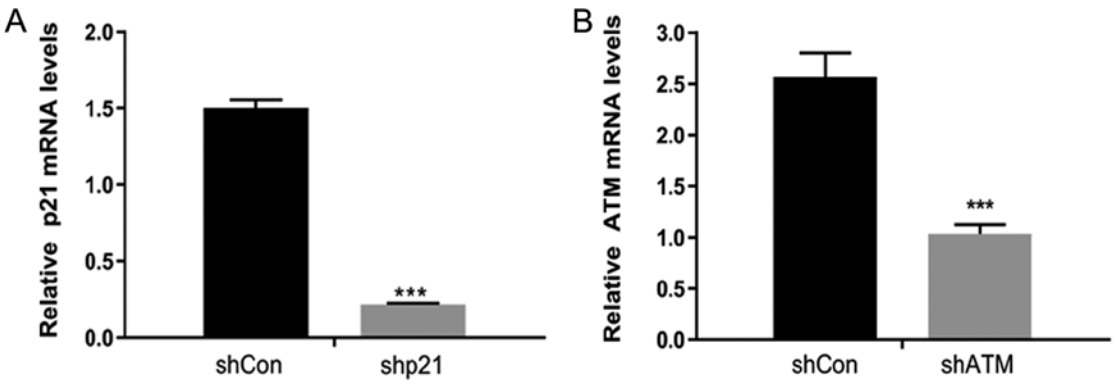

Figure 1. Confirmation of successful transfection of shp21 and shATM into HK-2 cells. (A and B) Reverse transcription quantitative-PCR was used to analyze the expression levels of (A) p21 and (B) ATM following transfection with shp21 or shATM, respectively. n=3; ***P<0.001 vs. shCon. ATM, ataxia telangiectasia mutated; Con, control; sh, short hairpin RNA.

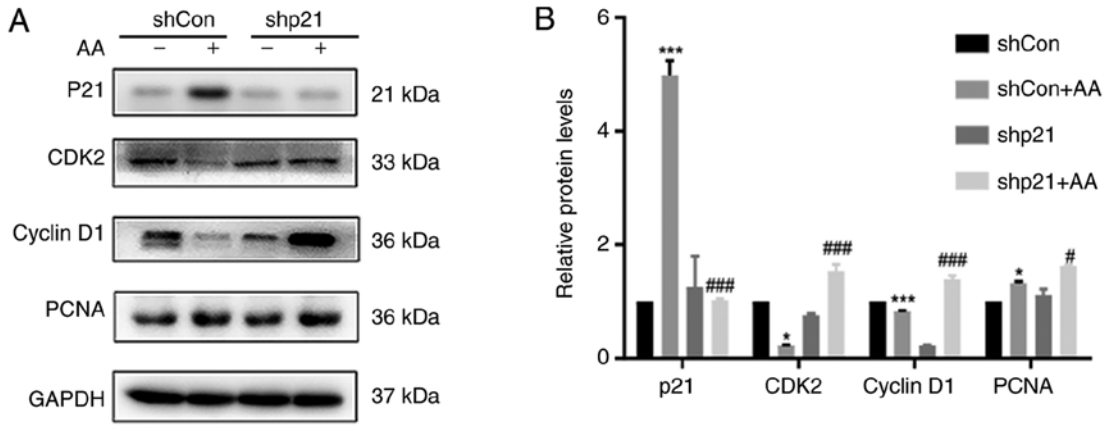

Figure 2. Knockdown of p21 promotes cell cycle progression and cell proliferation. (A) Western blotting was used to analyze the expression levels of p21, CDK2, cyclin D1 and PCNA in cells transfected with shp21 or shCon with or without AA-induced injury. (B) Semi-quantification of data from. (A) n=3; *P<0.05, ***P<0.001 vs. shCon; #P<0.05, ###P<0. 001 vs. shCon + AA. AA, aristolochic acid; Con, control; PCNA, proliferating cell nuclear antigen; sh, short hairpin RNA.

significantly reduced the protein ratio of p-Chk2/Chk2, p-p53/p53 and the protein expression level of r-H2AX compared with the shCON +AA group (Fig. 5). In addition, AA-induced shCon group significantly increased the protein expression levels of p21 compared with non-AA treated shCon group, which would result in cell cycle arrest (Fig. 6); however, following ATM knockdown, the expression levels of p21, CDK2 and cyclin D1 were significantly decreased, thereby promoting the cell cycle process.

shRNA knockdown of ATM expression levels promotes proliferation, increases caspase-3 activity and induces apoptosis in AA-induced HK-2 cells. To further investigate whether DDR signaling affects the proliferation and apoptosis of AA-induced HK-2 cells, the protein expression levels of PCNA were analyzed using western blotting (Fig. 6A and B), the proportion of apoptotic cells were determined using flow cytometry (Fig. 7A and B) and caspase-3 activity was analyzed using a caspase-3 activity kit (Fig. 7C). It was found that cells in the shATM + AA group exhibited significantly increased expression levels of PCNA and increased apoptotic rates compared with the shCon + AA group in the presence of AA damage.

Discussion

During the vicious cycle of cell proliferation and death, the signaling pathways within cells exist in a contradictory state; that is, proliferative signaling pathways and cell death signaling pathways simultaneously exist (10). Renal injury has been demonstrated to stimulate the surviving RTECs to enter the cell cycle; however, this has been observed to increase their sensitivity to the external environment and subsequently promote their cell death (17), hence the formation of the 'proliferation-death' cycle. Following the persistence of injury-related factors, the inhibition of proliferation through the mTOR and hedgehog signaling pathways has been found to protect cells against injury factors (27,28). At the root of TIF development should be the injury caused by the vicious cycle; however, inflammation, invasion, RTEC transdifferentiation and myofibroblast proliferation are only the intermediate links. Thus, it was hypothesized that the proliferation-death in RTECs may promote the progression of TIF following the persistence of injury-related factors. The present study revealed that the DDR was involved in the aberrant proliferation and cell death cycle, in addition to suggesting that the p21 protein may serve a major role in the 'proliferation-death' cycle.

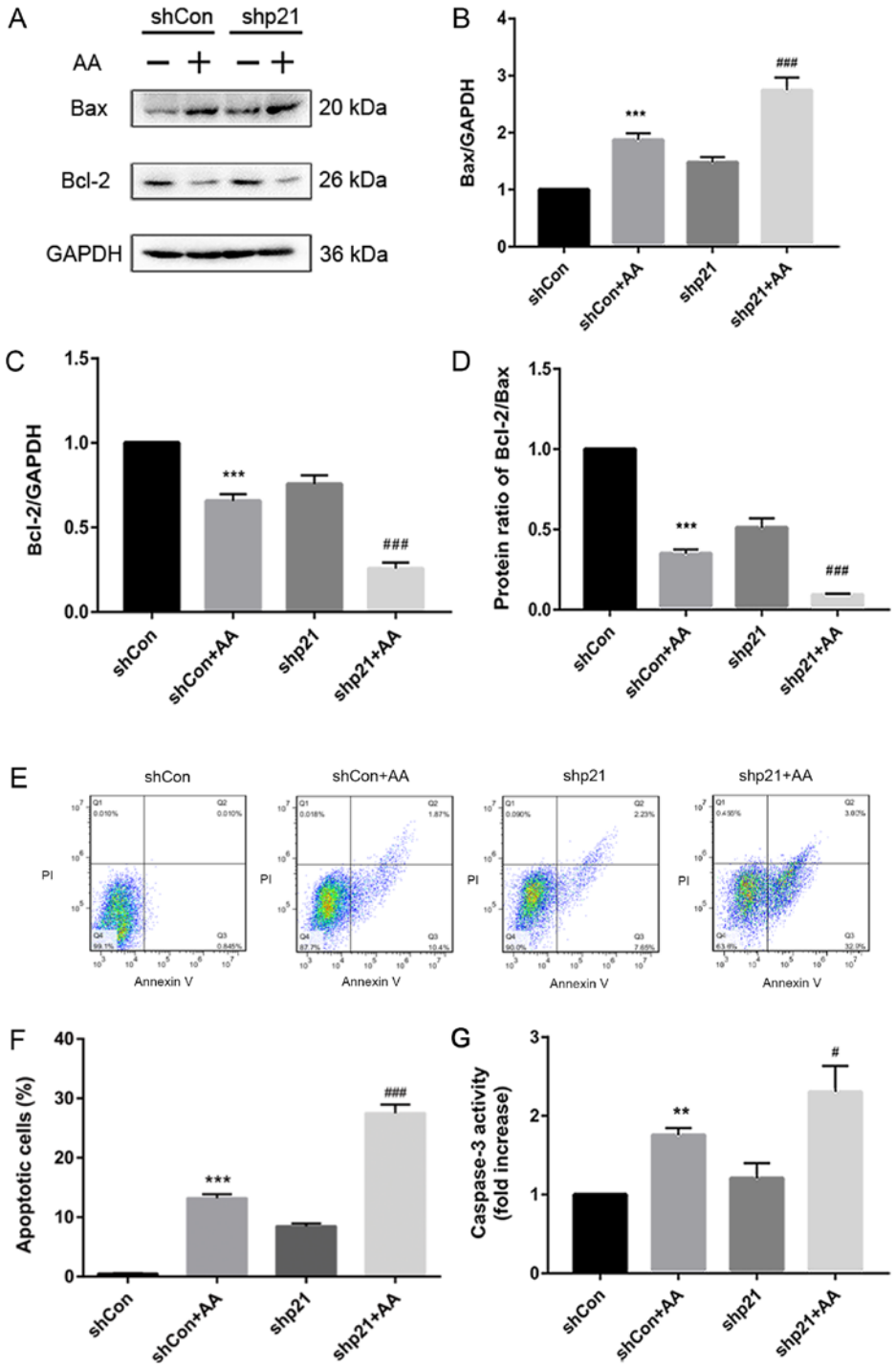

Figure 3. Genetic knockdown of p21 increases AA-induced rates of apoptosis in HK-2 cells. (A) Bax and Bcl-2 protein expression levels were detected using western blotting in cells transfected with shp21 or shCon with or without AA-induced injury. (B-D) Semi-quantification of (B) Bax, (C) Bcl-2 and (D) Bcl-2/Bax protein expression levels from (A). (E) Flow cytometric analysis of apoptosis was performed in cells transfected with shp21 or shCon with or without AA-induced injury. Following the stimulation of cells with AA for 24 h, cells were collected and stained with annexin V-FITC and propidium iodide. (F) Quantification of data from (E). (G) Caspase-3 activity was detected in cells transfected with shp21 or shCon with or without AA-induced injury using a caspase-3 activity kit. All data are presented as the mean ± standard error of the mean; n=3; **P<0.01, ***P<0.001 vs. shCon; $^{\#}$P<0.05, $^{\#\#\#}$P<0.001 vs. shCon + AA. AA, aristolochic acid; Con, control; sh, small hairpin RNA.

Renal fibrosis is the pathological hallmark of chronic kidney disease and it manifests as glomerulosclerosis and tubulointerstitial fibrosis (29). Podocyte loss and dysfunction in the glomerulus, in addition to tubular epithelial cell atrophy and loss, has also been reported to contribute to chronic kidney disease (30). Thus, the present study investigated the effect of AA-induced chronic injury in human RTECs by interfering with the expression levels of ATM and p21 proteins.

p21 regulates various p53-dependent and p53-independent cell functions; in addition to regulating the cell cycle, p21 regulates apoptosis, induces senescence and maintains cellular quiescence in response to various stimuli, including drugs, blood loss, infection, or exposure to cytotoxic agents (31).

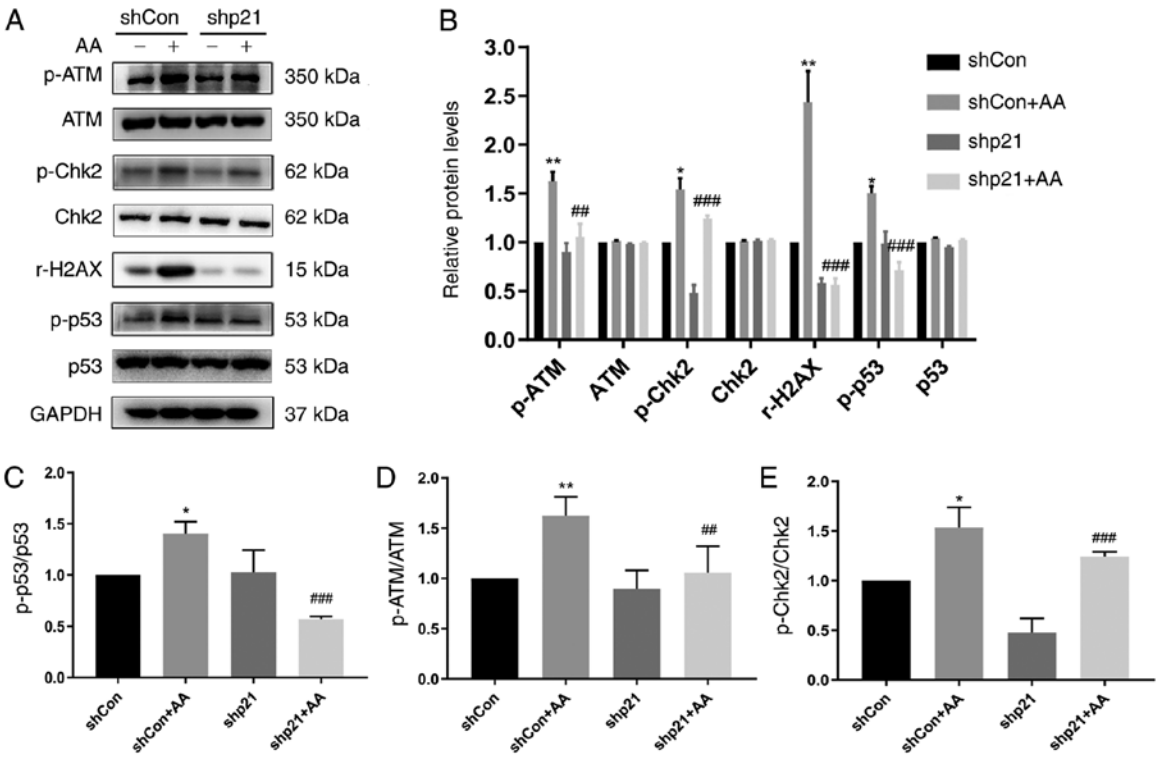

Figure 4. p21 knockdown reduces the DNA damage response in HK-2 cells. (A) Protein expression levels of p-ATM, ATM, p-Chk2, Chk2, rH2AX, p-p53 and p53 were analyzed using western blotting in cells transfected with shp21 or shCon with or without AA-induced injury. (B) Semi-quantification of data from (A). (C-E) Phosphorylated/total protein expression ratios of (C) p-p53/p53, (D) p-ATM/ATM and (E) p-Chk2/Chk2 from data from. (A) Data are presented as the mean ± standard error of the mean; n=3; *P<0.05, **P<0.01 vs. shCon; $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. shCon + AA. AA, aristolochic acid; ATM, ataxia telangiectasia mutated; Chk2, checkpoint kinase 2; Con, control; p, phosphorylated; r-H2AX, H2AX phosphorylation at Serine 139; sh, short hairpin RNA.

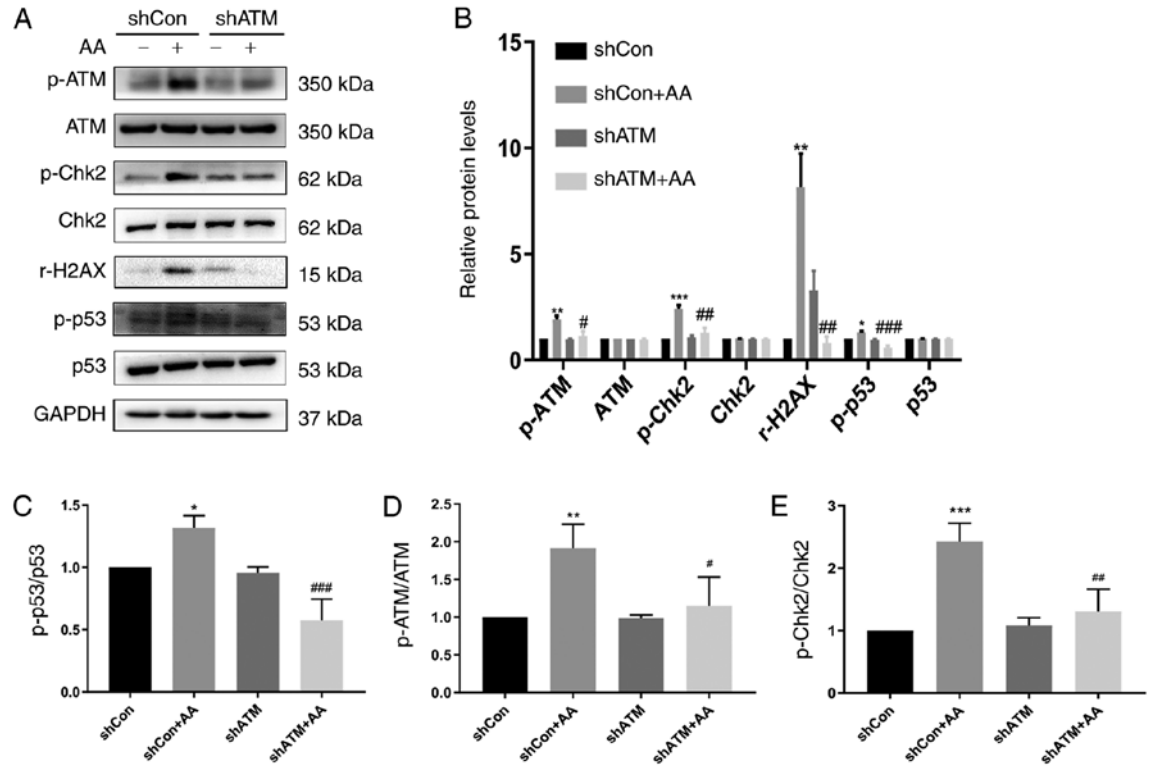

Figure 5. Genetic knockdown of ATM reduces the DNA damage response and p-p53 expression levels. (A) Protein expression levels of p-ATM, ATM, p-Chk2, Chk2, r-H2AX, p-p53 and p53 were detected using western blotting in cells transfected with shATM or shCon with or without AA-induced injury. (B) Semi-quantification of data from (A). (C-E) Phosphorylated/total protein expression ratio of (C) p-p53/p53, (D) p-ATM/ATM and (E) p-Chk2/Chk2 from data from. (A) Data are presented as the mean ± standard error of the mean; n=3; *P<0.05, **P<0.01, ***P<0.001 vs. shCon; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. shCon + AA. AA, aristolochic acid; ATM, ataxia telangiectasia mutated; Con, control; Chk2, checkpoint kinase 2; p, phosphorylated; r-H2AX, H2AX phosphorylation at Serine 139; sh, short hairpin RNA.

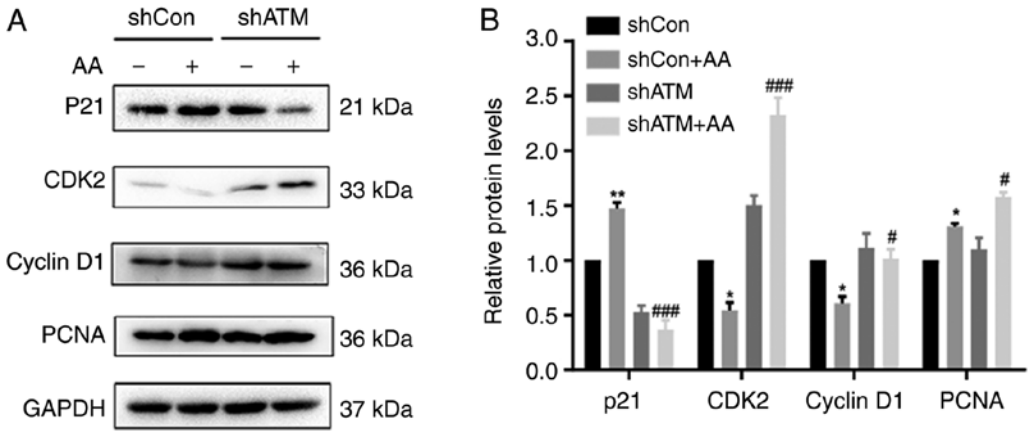

Figure 6. Genetic knockdown of ATM suppresses p21 activation, induces cell cycle arrest and promotes the proliferation of HK-2 cells. (A) Protein expression levels of p21, CDK2, cyclin D1 and PCNA were analyzed using western blotting in cells transfected with shATM or shCon with or without AA-induced injury. (B) Semi-quantification of data from. Data are presented as the mean ± standard error of the mean; n=3; *P<0.05, **P<0.01 vs. shCon; $^{\#}$P<0.05, $^{\#\#\#}$P<0.001 vs. shCon + AA. AA, aristolochic acid; ATM, ataxia telangiectasia mutate d; Con, control; PCNA, proliferating cell nuclear antigen; sh, short hairpin RNA.

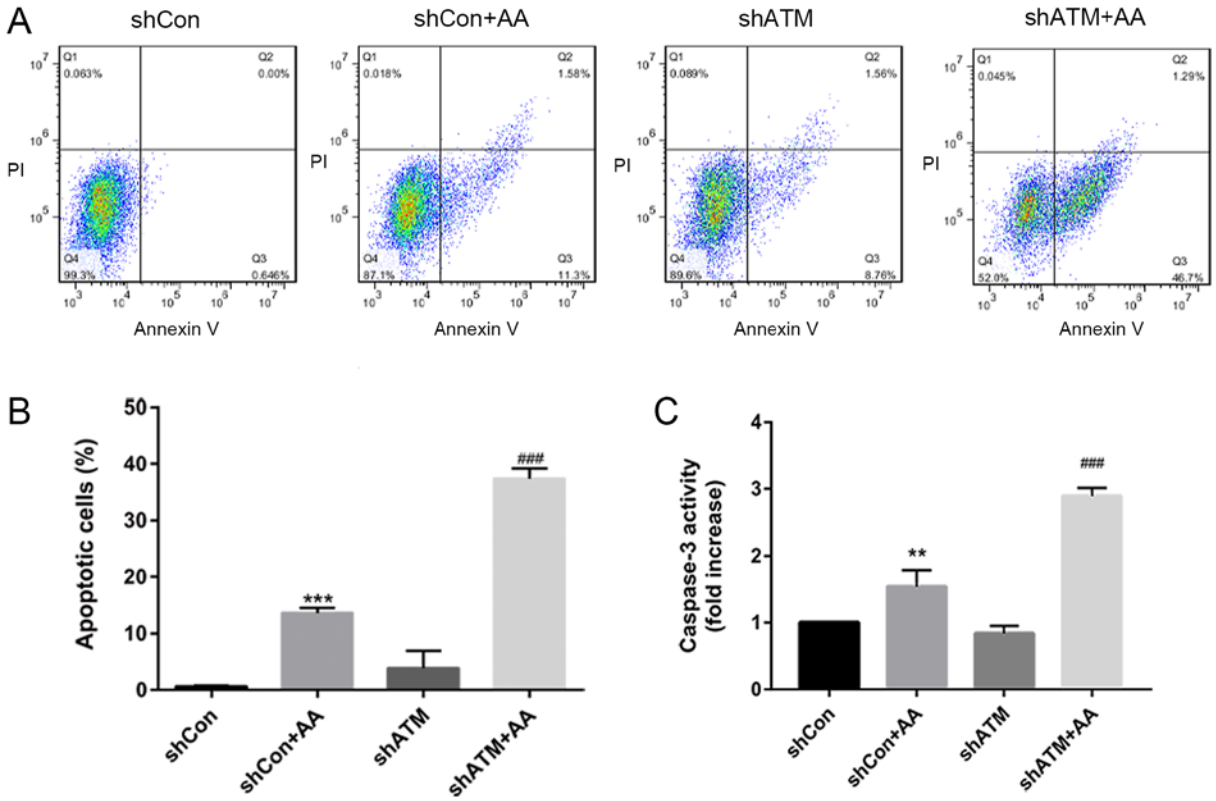

Figure 7. Genetic knockdown of ATM induces apoptosis and increases caspase-3 activity. (A) Flow cytometric analysis of apoptosis in cells transfected with shATM or shCon with or without AA-induced injury. (B) Semi-quantification of apoptosis from part A. (C) Caspase-3 activity was analyzed using a caspase-3 activity kit in cells transfected with shATM or shCon with or without AA-induced injury. Data is presented as the mean ± standard error of the mean (n=3). **P<0.01,***P<0.001 vs. shCon group; $^{\#\#\#}$P<0.001 vs. shCon + AA group. sh, small hairpin RNA; Con, control; AA, aristolochic acid; ATM, ataxia telangiectasia mutated.

Alongside binding to cell cycle proteins/CDK complexes, p21 has also been discovered to contain the COOH terminal binding site of PCNA (32,33), which has been found to serve important roles in DNA replication and different types of DNA repair, including nucleotide excision repair, mismatch repair and base excision repair (34,35). Moreover, p21 has been revealed to interact directly with PCNA and block DNA synthesis through DNA polymerase δ (36). It has also been observed to regulate DNA repair through its interaction with PCNA and related proteins (37). The mechanisms of p21-induced inhibition over cell death, including inhibiting the promoter caspase cleavage have been investigated previously (38); the interaction of p21 with procaspase-3 resulted in the resistance to Fas-mediated cell death and the stabilization of the apoptosis regulator cellular inhibitor of apoptosis protein 1 (39,40), whereas p21 overexpression prevented the cytoplasmic domain-induced caspase-8 cleavage and death receptor 4 (DR4)-CD-induced apoptosis of the DR4 receptor (38). In addition, p21 has been discovered to

contain the amino-terminus that interacts with procaspase-3 and suppresses its activation by inhibiting its conversion to the active protease (41). Similar to previous reports, the results of the present study demonstrated that in the AA-treated, p21 knocked down HK-2 cells, the inhibitory effect over CDK2 and cyclin D1 was weakened, thus driving cell cycle progression and promoting proliferation. Furthermore, p21 knockdown in the injured cells stimulated caspase-3 expression and induced cell apoptosis, whilst promoting cell cycle progression and enhancing the sensitivity of cells to injury factors (such as AA). As expected, in the absence of the cell cycle inhibitor protein, p21, the proliferative activity, proportion of injured cells and the apoptotic rate were all increased. These findings highlighted the transition from cell proliferation to death and demonstrated that the acceleration of the cell cycle may affect the formation and extent of the proliferation-death cycle. The present study showed that p21 knocked down HK-2 cells drove cell cycle progression and promoted proliferation, stimulated caspase-3 expression and induced cell apoptosis.

ATM regulates cellular DNA repair and serves an important role in maintaining chromosomal integrity and genome stability (42). DNA damage during the cell cycle has been demonstrated to activate ATM/ataxia telangiectasia and Rad 3-related (ATR) and their downstream kinases, Chk2 and Cdc25 family members, which are involved in the checkpoint pathway; this enabled cell proliferation to be halted until damage is repaired (43), which often involves a series of proteins, such as BRCA1/γH2AX/E2F1/RAD (44). However, if the damaged DNA cannot be repaired, the accumulation of activated ATM/ATR has been found to rapidly phosphorylate the p53 protein at the Ser15 site and activate p53 (45). Activated p53 has been observed to further induce apoptosis in p53 upregulated modulator of apoptosis/NOXA/Bax-mediated mitochondrial pathways (46) and this process is linked to proliferation and death (19). ATM serves a central role in phosphorylating DDR and regulating cell cycle-related molecules throughout the entire process (47). In the current study, acute injury in the HK-2 cells promoted ATM signal activation and the activation of the DDR. In brief, acute injury increased the expression levels of ATM/p53 to activate apoptosis and p53 subsequently activated p21 to promote cell cycle arrest. Interestingly, p53 and p21 expression levels decreased following the genetic knockdown of ATM, thus causing increased expression levels of CDK2 and cyclin D1; and accelerated cell cycle, promoted apoptosis and stimulated cell proliferation. The increased rate of apoptosis may be due to the fact that following the reduced expression levels of p21 in response to ATM knockdown, the effect of p21 was increased and the cells lost their p21-induced anti-apoptotic effect, leading to increased apoptosis.

In conclusion, the present study confirmed that cell death occurs during the progression of the cell cycle. The genetic knockdown of p21 was found to increase cell cycle progression, promote proliferation and cause cell death. In addition, although proliferation and apoptosis could occur at the same time, it could also occur periodically. It was clear that early diseases mostly began with proliferation, so it was investigated that as the course of the disease proceeded, whether this pattern occurred periodically all the time. Thus, the present study discovered that the vicious cycle of proliferation and death may be initiated through the DDR signaling pathway. ATM, as a crucial molecule of the DDR, has been found to serve an important role in the regulation of persistent chronic injury (48,49). In the present study, the genetic knockdown of ATM promoted apoptosis and increased proliferation. The increase in apoptosis was hypothesized to be due to the decreased expression levels of p21 caused by the genetic knockdown of ATM. Thus, the regulatory crosstalk between the ATM protein and p21 protein were suggested to serve an important role in the proliferation-death cycle. These findings provided a potential method for further pathophysiological research into the process of chronic injury.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

BC made substantial contributions to the conception and design of the study; LS, XZ JL and CW performed the experiments, data analysis and interpretation; and XZ and CW were responsible for drafting the article and critically revising it for important intellectual content. LS, XZ and JL contributed equally to this article. All authors read and approved the final manuscript. All authors are accountable for all aspects of the study in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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