Hepatitis B virus X protein activates the ATM–Chk2 pathway and delays cell cycle progression

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Genetic instability is intimately associated with tumour development. In particular, liver cancers associated with hepatitis B virus (HBV) exhibit high genetic instability; however, our understanding of the underlying molecular mechanisms remains limited. In this study, we found that γ-H2AX, a marker of DNA double-strand breaks (DSBs), and the levels of phospho-Chk2 (p-Chk2, the activated form) were significantly elevated in HBV-associated hepatocellular carcinomas and neighbouring regenerating nodules. Likewise, introduction of the pHBV or pMyc-HBx plasmids into cells induced accumulation of γ-H2AX foci and increased the p-Chk2 level. In these cells, inhibitory phosphorylation of Cdc25C phosphatase (Ser216) and CDK1 (Tyr15) was elevated; consequently, cell-cycle progression was delayed at G2/M phase, suggesting that activation of the ATM–Chk2 pathway by the HBV X protein (HBx) induces cell-cycle delay. Accordingly, inhibition of ataxia telangiectasia mutated (ATM) by caffeine or siRNA abolished the increase in the p-Chk2 level and restored the delayed CDK1 kinase activity in ChangX cells. We also found that cytoplasmic HBx, but not nuclear HBx, induced reactive oxygen species (ROS) production and led to the accumulation of γ-H2AX foci and the increased p-Chk2 level. Together, these data indicate that HBx-induced ROS accumulation induces DNA damage that activates the ATM–Chk2 pathway. Our findings provide insight into the mechanisms of HBV pathogenesis.

INTRODUCTION

Hepatocellular carcinoma (HCC) is a leading cause of death worldwide, and one of the primary aetiological factors for the development of liver cancer is persistent infection by hepatitis B virus (HBV) or hepatitis C virus. The HBV genome encodes polymerase, surface antigen, core protein and HBV X protein (HBx), a non-structural regulatory protein. HBx is involved in the development of HBV-associated liver cancer through modulation of various cellular activities including transcriptional activation (Bouchard et al., 2001; Klein & Schneider, 1997), signal transduction (Miao et al., 2006; Shih et al., 2003) and epigenetic modification of the host genome (Zhang et al., 2013).

Genetic instability plays a crucial role in cancer initiation and malignant progression. In particular, tumours associated with HBV infection are characterized by high genetic instability, especially in specific regions of chromosomes (Laurent-Puig et al., 2001). Increased genetic instability in HBV-related tumours may be triggered by insertion of the viral genome into host chromosomes (Amaddeo et al., 2015; Bréchot, 2004; Dejean et al., 1986; Wang et al., 1990). Alternatively, HBx protein itself may increase...
the genetic instability in host cells, possibly by amplifying the centrosome numbers (Forgues et al., 2003; Kim et al., 2008; Yun et al., 2004). In addition, HBx binds HBXIP, which plays an important role in centrosome duplication and microtubule stability in mitosis (Wen et al., 2008). These activities of HBx result in aberrant mitotic spindle formation and chromosome missegregation, thereby increasing the risk of aneuploidy.

Furthermore, liver cells chronically infected with viruses are exposed to an inflammatory microenvironment for long periods of time. Under these conditions, liver cells can be exposed to constant DNA damaging agents such as free oxygen radicals or other extrinsic factors such as therapeutic drugs or ionizing radiation. These agents cause various types of DNA damage, including DNA double-strand breaks (DSBs) and excision of nucleotides or bases. To cope with DSBs, cells employ the G2 DNA-damage checkpoint pathway. DSBs result in activation of the ATM (ataxia telangiectasia mutated) and ATR (ATM- and Rad3-related) kinases, which phosphorylate multiple downstream targets including H2AX, Chk2, and repair factors. Many viruses have evolved to cope with DNA damage or interfere with host cell DNA-damage response/repair pathways (Martin-Lluesma et al., 2008), thereby affecting host genome stability. At present, it remains unclear whether HBx is involved in the G2 DNA-damage checkpoint.

In this study, we investigated whether the G2 DNA-damage checkpoint system is activated during HBV-mediated pathogenesis. We found that γ-H2AX and phospho-Chk2 (p-Chk2) levels were elevated in HBV-related liver cells and hepatocellular carcinoma specimens. Moreover, HBx-induced reactive oxygen species (ROS) accumulation induced DNA damage that activated the ATM–Chk2 pathway. Together, these effects may increase genetic instability and contribute to multi-step carcinogenesis.

### RESULTS

#### DNA DSBs occur in HBV-associated hepatocellular carcinomas and regenerating nodules

Genetic instability is linked to tumour development and malignant progression, and DSBs of DNA are a major cause of genetic instability. In this study, we monitored the levels of γ-H2AX and p-Chk2, both of which are induced by DSBs, in liver cancer patients. We performed immunohistochemistry on specimens of paraffin-embedded HCC and adjacent regenerating nodules obtained from HBV-infected cancer patients. In each slide, ten random sites were selected for microscopic observation. In ten normal liver tissues, no nuclei were stained positively by anti-γ-H2AX and anti-p-Chk2 antibodies. By contrast, 40–60 % of HCC patients exhibited positive staining for both phosphoproteins in regenerating nodules and tumour tissue (Figs 1a, b, and S1 and S2 available in the online Supplementary Material). The proportion of γ-H2AX and p-Chk2 positive cells increased as disease progressed (Fig. 1c). Thus, DSB formation is ongoing in HBV-infected liver tissue.

#### Expression of HBx in cells increases the proportion of γ-H2AX-positive cells

HBx plays a crucial role in the pathogenesis of HCC. Hence, we next examined whether accumulation of γ-H2AX foci in liver specimens is directly linked to HBx. First, we monitored γ-H2AX staining in ChangX cells that were originally established as an inducible system expressing HBx protein (Yun et al., 2000). During continuous passages, HBx protein is detected in the absence of doxycycline in these cells (Chae et al., 2013; Cho et al., 2014). We found that half of the cells examined were strongly positive for γ-H2AX (Fig. 2a). Next, we transiently introduced the HBx gene into Chang cells, and found that 24.7 % of HBx-transfected cells exhibited γ-H2AX foci after 48 h. Original Chang cell clones with hepatic characteristics were selected through serial dilution and used for all experiments (Fig. S3). We confirmed albumin expression in both Chang and Huh7 cells whereas the L1 retrotransposon appeared only in the HeLa cells. The L1 retrotransposon marker was discovered as a HeLa-specific diagnostic marker to discriminate HeLa contamination (Rahbari et al., 2009). We confirmed our findings in pHBV-transfected cells. Specifically, we transfected an HBV genome plasmid (pHBV) into Chang cells, which were then subjected to staining for γ-H2AX 48 h later. DNA damage occurred in approximately 20 % of cells transfected with pHBV (Fig. 2b). However, when cells were transfected with an HBx-defective HBV genome (pHBV-X; translation start codon of HBx was disrupted), the proportion of γ-H2AX positive cells was significantly reduced (22.6 % vs 11.7 %, **P<0.01). We verified these findings by immunoblotting for γ-H2AX levels, revealing that γ-H2AX levels were elevated by pHBV in a dose-dependent manner (Fig. 2c). However, we noticed that γ-H2AX-positive cells still remained even in the absence of HBx expression (Fig. 2b, c). Thus, we interpreted that γ-H2AX-positive cells in pHBV-Xcells may come from HBV surface antigen (HBs) expression because it was recently reported that HBs could also induce DNA damage (Chung, 2013). Together, our data demonstrate that HBx is one of the major factors causing DSBs in HBV-related liver cells.

#### Activation of the ATM–Chk2 pathway by HBx induces delays in the cell cycle at the G2/M transition

ATM and ATR are the master kinase sensors responding to different types of DNA damage (Shiloh, 2001). They activate the transducer kinases Chk1 and Chk2, which phosphorylate downstream molecules including p53 and the Cdc25 family proteins. These effector proteins in turn control the cell cycle by governing the fate decision between cell-cycle arrest and apoptosis (Chen & Poon, 2008;
Taylor & Stark, 2001). Because the level of γ-H2AX was increased in HBx-expressing cells and human liver tissues, we investigated whether HBx activates the ATM–Chk2 signalling cascades. To test this, we transfected the HBx gene and determined the p-Chk1 and p-Chk2 levels. It is known that ATM phosphorylates the Thr68 of Chk2 upon DNA damage whereas ATR phosphorylates the Ser345 of Chk1 at stalled replication forks (Bartek & Lukas, 2003). We found that the p-Chk2 level was increased 48 h after HBx expression whereas the p-Chk1 levels remained unchanged by HBx expression (Fig. 3a). To verify whether the ATM is the upstream kinase of p-Chk2, siRNA against

**Fig. 1.** DNA damage signal increases in HBV-associated hepatocellular carcinomas. (a) Immunohistochemical analysis of γ-H2AX and p-CHK2 in liver cancer patients. (b, c) Bar graphs denote percentage of cells positive for γ-H2AX or p-CHK2. Data are shown as means ± SEM. *P<0.1, **P<0.05, ***P<0.001 by Student’s t-test. R.N., regenerating nodule.
ATM or ATR was applied to HBx-transfected cells and the effect on p-Chk2 level was monitored. As shown in Fig. 3(b), the elevated p-Chk2 level was abolished by ATM siRNA but not ATR siRNA, suggesting that the increase in the p-Chk2 level induced by HBx is activated by ATM kinase but not ATR. In addition, we investigated whether

Fig. 2. γ-H2AX-positive cells were increased upon HBx expression. (a) At 48 h post-transfection with pMyc and pMyc-HBx vectors, cells were fixed with acetone/methanol (1 : 1, v/v) and stained with anti-γ-H2AX antibody and visualized with FITC-conjugated secondary antibody (green). Nucleus was stained with propidium iodide (PI) (red). Image was obtained by confocal laser scanning microscopy (CLSM) and γ-H2AX-positive cells were randomly counted from more than 200 cells in each case. Each bar denotes mean ± SD from at least five independent experiments. Scale bars, 5 μm. (b) HBV replicon (pHBV) containing whole HBV viral DNA or pHBV-X− (HBV replicon with mutated translation start codon of HBx) were transfected into Chang cells. γ-H2AX signals were visualized using FITC-conjugated secondary antibody (green). HBx was stained with anti-HBx antibody and visualized with cy3 conjugated secondary antibody (red) and nucleus was stained with DAPI (blue). Each bar denotes mean ± SD from three independent experiments. **P<0.05 by Student’s t-test. Scale bars, 5 μm. (c) pMyc, pMycX, pHBV or pHBV-X− constructs were transfected to Chang cells. After 48 h of incubation, Chk2 was analysed by Western blot analysis.
Fig. 3. HBx induces G₂/M delay through ATM-Chk2 pathway activation. (a) Cells were transfected with pMyc or pMycX and incubated for the times indicated. The cell lysates were resolved by SDS-PAGE and immunoblotted with anti-Cdc25 (Ser21), anti-Chk2 (Thr68) and anti-Cdk1 (Tyr15) antibodies. (b) Cells were transfected with ATR siRNA or ATM siRNA and co-transfected with pMycX for 48 h. Cell lysates were subjected to immunoblot analysis. (c) Cells were synchronized at G₁/S boundary using the DTB method. After the first thymidine block, cells were transfected with myc-tagged HBx (pMycX) or control constructs (pMyc) respectively. Release from the DTB was set as T₀. At the indicated times after DTB release, cells were harvested for FACS analysis. (d) The parental Chang cells and HBx-expressing ChangX cells were synchronized at the G₁/S phase by the DTB method. After synchronization, cells were released from the DTB and harvested for analysis of mitotic indices using immunoblot. To determine the cyclin B1/Cdk1 and cyclin A/Cdk2 activities, antibodies for cyclin B or cyclin A were used for the immunoprecipitation of cyclin B1/Cdk1 or cyclin A/Cdk2 complexes. Histone H1 was used as a substrate. (e) Cells stably expressing or not expressing HBx were synchronized at the G₁/S phase by the DTB method, and at 8 h after DTB release, DMSO, 2 mM caffeine or 5 μM SB203580 were applied to the cells. Cells were harvested for the cyclin B1/Cdk1 IP-kinase assays at the times indicated.
activation of the ATM–Chk2 pathway by HBx affects cell-cycle progression. At the G2/M transition, CDK1 activity is stimulated by dephosphorylation at Tyr15, a reaction catalysed by the Cdc25C phosphatase (Pinsky & Biggins, 2005). Upon DNA damage, active Chk2 phosphorylates the Ser216 residue of Cdc25C, and the phosphorylated Cdc25C loses the ability to remove the phosphate from Tyr15 of CDK1 (Darbon et al., 2000), thereby inhibiting cell-cycle progression. When we determined the inhibitory phosphorylation of Cdc25C (Ser216) in HBx-expressing cells, we also found that p-Cdc25C levels increased along with p-Chk2 levels (Fig. 3a). In addition, the p-Tyr15 of CDK1 level remained elevated in HBx-transfected cells (Fig. 3c, right panel). Together, these data suggest that activation of the ATM–Chk2 pathway by HBx affects cell-cycle progression. To further investigate the effect of HBx on cell-cycle progression, we transfected Chang cells with pMyc or pMyc-HBx vector and synchronized them at the G1/S boundary using a double thymidine block (DTB) method. The time of release from the DTB was defined as time zero (T=0). DNA profiles of cells were analysed by FACS at the times indicated (Fig. 3c). Eight hours after release from the DTB, the majority of cells in the control and HBx-transfected groups had progressed into G2/M phase (83.99 % vs 78.78 %). After 12 h, the percentage of cells in G2/M phase had significantly decreased (to 22.61 %) in controls, but remained high (52.1 %) in HBx-transfected cells. The changes in cell-cycle profiles were verified by the levels of cyclin A and B, as well as kinase activities of CDK1 and CDK2 (Fig. 3d). Consistent with the cell-cycle profiles, CDK1 activity in ChangX cells peaked at 12 h after DTB release, a delay of 2 h relative to Chang cells. Restoration of CDK2 activity was also delayed in ChangX cells. As with CDK1 and CDK2 kinase activities, degradation of cyclin A and B was delayed in ChangX cells. We predicted that if the cell-cycle delay induced by HBx is caused by the ATM–Chk2 pathway, inhibition of ATM by caffeine would rescue this delay in cell-cycle progression. In this experiment, we added SB203580, an inhibitor of p38 MAPK, because HBx induces p38 activation (Tarn et al., 2002) and active p38 kinase is involved in the DNA-damage response (Goldstone et al., 2001; Mikhailov et al., 2004). We found that CDK1 kinase activity in ChangX cells peaked at 12 h after DTB release, whereas addition of caffeine (as an ATM inhibitor) caused the peak to occur earlier, at 9 h (Fig. 3e). By contrast, addition of SB203580 did not alter the cell-cycle profile of ChangX cells. Taken together, these data suggest that HBx activates the ATM–Chk2 signalling cascade, thereby inducing cell-cycle delay in G2/M phase.

**HBx increases cellular ROS levels, which correlate with activation of DNA-damage responses**

Next, we investigated the mechanism by which HBx protein in liver cells induces DNA damage. Because HBx induces oxidative stress in liver cells, we hypothesized that HBx causes DNA damage through ROS generation. As previously shown by our group and others (Lim et al., 2010; Wang et al., 2003; Waris et al., 2001), transfection of pMyc-HBx into Chang cells increased cellular ROS levels in a time-dependent manner (Fig. 4a, left). Furthermore, this elevation in ROS levels was accompanied by an increase in the p-Chk2 level (Fig. 4a, right). HBx localizes in the nucleus, cytoplasm or mitochondria depending on its expression level, and its subcellular localization is important for its pleotropic effects at various stages of carcinogenesis (Henkler et al., 2001; Ma et al., 2011). Therefore, we employed two retargeting mutants of HBx, pHBx-NLS and pHBx-NES, which, respectively, encode HBx containing a nuclear localization signal (NLS) or a nuclear export signal (NES) at the N terminus. pHBx-NLS-transfected cells exhibited lower DCF-DA fluorescence intensity than NES-HBx-transfected cells (Fig. 4b). As expected, HBx-NLS localized only in the nucleus, whereas HBx-NES mainly localized in the cytoplasm (Fig. 4c). Notably, 24 % of pHBx-NES-transfected cells exhibited positive immunostaining for γ-H2AX. By contrast, only a small portion (8.5 %) of pHBx-NLS-transfected cells were positive for γ-H2AX (Fig. 4c). Consistent with this, we observed that the p-Chk2 level was higher in cells transfected with pHBx-NES than in those transfected with pHBx-NLS (Fig. 4c). Together, these results suggest that ROS contributes to HBx-induced DNA damage.

**DISCUSSION**

In this study, we demonstrated that HBx causes DSBs in the host genome. To cope with these DSBs, host cells employ the ATM–Chk2 checkpoint pathway, thereby delaying cell-cycle progression. In addition, HBx-mediated ROS generation is one of the causes of DNA damage. γ-H2AX foci disappear once DNA breaks are sealed (Banáth et al., 2004) and γ-H2AX foci were shown to persist in 65 % of the HCC specimens, suggesting that DSBs in HCC specimens are not fully repaired. It is noteworthy that mutations in NBS1 (Nijmegen breakage syndrome gene 1), one of the major components in the G2 DNA-damage checkpoint pathway, were reported in HCC (Wang et al., 2013). Thus, DSBs in HBV-related tumours represent another causative factor contributing to genetic instability. DNA-damage checkpoint activation by a viral oncoprotein is not unique to HBx. In 2003, Haoudi et al. (2003) reported that the Tax protein from human T-cell leukemia virus type I induces G2 arrest through the activation of Chk2. Tax interacts with Chk2 and induces relocalization of the DNA-damage sensor 53BP1 in an ATM-dependent manner (Haoudi et al., 2003). In addition, human immunoodeficiency virus type 1 Vpr also arrests infected cells in G2 phase via the activation of ATR. Vpr utilizes a cellular signalling pathway whose physiological function is recognition of replication stress, such as γ-H2AX and BRCA1 (Zimmerman et al., 2004). On the other hand, Tarn et al. (2002) suggested that HBx could arrest AML12-derived...
cells at G2/M via the p38 MAPK pathway. However, we could not detect p38 activity in ChangX cells, and treatment with a p38 inhibitor did not rescue the G2 delay (Fig. 3). Thus, in our system, the ATM–Chk2 checkpoint system is the predominant pathway responding to DNA damage.

In human livers, expression levels of HBx protein are generally low (Park et al., 2003). The subcellular localization of HBx is primarily cytoplasmic, with a small fraction in the nucleus. However, high levels of HBx expression lead to abnormal distribution of mitochondria. The dynamic distribution of HBx may be important to the multiple functions of HBx at different stages of the HBV life cycle (Henkler et al., 2001; Ma et al., 2011). We found that cytoplasmic HBx (HBx-NES) could induce ROS and γ-H2AX foci formation, whereas nuclear HBx (HBx-NLS) could not. We previously reported that mitochondria-targeted HBx is capable of generating ROS (Lim et al., 2010). Thus, mitochondria-generated ROS may be a major source of DNA breaks in HBV-related liver cells.

**Fig. 4.** HBx-induced DNA damage is correlated with ROS generation. (a, b) Cells were transfected with pMyc or pMycX and cellular ROS levels were determined by DCF-DA, a ROS-specific dye, using FACS analysis. Cells were preincubated with DCF-DA for 10 min and washed with PBS. Trypsinized cells were analysed by FACS immediately. Mean ± SD from at least seven independent experiments. *P < 0.05 by student’s t-test. (c) HBx-NLS- or HBx-NES-transfected cells were fixed with acetone/methanol (1:1, v/v) solution and flag-tagged HBx was stained with flag antibody. HBx was shown as red and the nucleus was stained with DAPI, blue. γ-H2AX immunostaining was performed after NLS-HBx or NES-HBx transfection and γ-H2AX foci positive cells were counted. Mean ± SD from three independent experiments. Scale bars, 5 μm.
METHODS

Materials. Caffeine was purchased from Sigma and p38 inhibitor, SB203580, was from Calbiochem. Antibodies against p-Chk2 (Thr68), Chk2, p-p53 (Ser15) and p-CDK1 (Tyr15) were obtained from Cell Signaling Technology. Cyclin A, B and myc antibodies were purchased from Santa Cruz laboratory and flag and actin antibodies were from Sigma. Antibodies against γ-H2AX were obtained from Upstate Technology. Alpha-tubulin antibody was from Oncogene.

Tissue samples and immunohistochemistry

Patient characteristics. Between June 2005 and May 2010, over 100 patients with HCC who underwent hepatectomy at Ajou University Hospital were enrolled in this study. A total of 100 paired specimens (tumour and surrounding non-tumour tissues) were obtained after hepatectomy. Of the 100 patients, 44 patients with available follow-up data were eligible for analysis of the association between clinico-pathological features and overall survival. All patients were chronic carriers of HBV. IRB (AJIRB-CRO-08-085).

Immunohistochemistry. Paraffin-embedded tissue sections were treated with 3% hydrogen peroxide and blocked with 5% BSA. The sections were then incubated sequentially with γ-H2AX and p-Chk2 antibodies on representative tissue sections in a Benchmark XT automated immunohistochemistry stainer (Ventana Medical System).

Cell culture and synchronization. HBx-transfected ChangX cells (Yun et al., 2000) and parental Chang (CCL-13, ATCC) cells were maintained in low-glucose Dulbecco’s minimal essential medium (DMEM) supplemented with 10% FBS (Invitrogen), respectively. Chang cell clones with hepatic characteristics (albumin production) were selected by serial dilution. ChangX cells were originally established by stably co-transfecting pTetX and pUHD172-1 as described previously (Yun et al., 2000). Cells were synchronized at the G1/S boundary by the DTB. Chang cells were treated with 2 mM thymidine (Sigma) for 18 h. After the first thymidine treatment, cells were washed with PBS twice and incubated with fresh medium for 6 h. Cells were then treated again with 2 mM thymidine for 18 h and washed with fresh medium for 6 h. Release from the DTB was set as T=0 (T0). After release from the DTB, the cells were allowed to grow under regular conditions for the times indicated.

Plasmids and transfection. pCMV-Myc (pMyc) and pCMV-MycHBx constructs were kindly provided by Dr Mi-Ok Lee (Seoul National University). Other HBx deletion mutants were made from pMyc-HBx by PCR using different sets of primers and subcloned into the pMyc construct. pHBV, pHBV-X-, pHBV-X-NLS and pHBV-X-NES constructs were kindly provided by Dr Wang-Shick Ryu (Yonsei University). The transfection of plasmids or siRNA oligonucleotides was carried out using polyethylenimine (PEI, Polsciences) and Lipofectamine 2000 (Invitrogen). Transfected DNAs or siRNA were allowed to express protein for an additional 48 h.

FACS analysis. Cells were synchronized at the G1/S boundary by using the DTB method. Upon release from the DTB, cells were allowed to grow for the times indicated. Cells were trypsinized and collected by centrifugation at 1500 r.p.m. for 3 min. Collected cells were washed with PBS and fixed with 70% (v/v) ethanol. After washing with PBS twice, cells were incubated with 1 μg RNase ml⁻¹ (Sigma) in PBS for 10 min at room temperature, followed by addition of 50 μg propidium iodide ml⁻¹ (Sigma) in PBS and analysed by using a FACS Vantage flow cytometer (Becton Dickinson).

Western blot analysis and CDK1/cyclin B1 kinase assay. Cells were lysed with RIPA buffer (10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 μg aprotinin ml⁻¹, 1 μg leupeptin ml⁻¹, 1 mM NaF and 5 mM Na3VO4) for 30 min on ice. After centrifugation at 11 000 r.p.m. for 30 min at 4°C, lystate was collected. The concentrations of lysesates were normalized by Bradford assay (Bio-Rad), and lysesates were analysed by immunoblotting. For immunoprecipitation-kinase assay, cell lysesates were prepared and CDK1 complexes were immunoprecipitated using anti-cyclin B1 antibody (Santa Cruz) and protein G Sepharose (Invitrogen). Immunopurified cyclin B1/CDK1 complexes were incubated in kinase buffer (20 mM HEPES, 0.14 M NaCl, 3 mM KCl, 5 mM MgCl2, pH 7.4) with 2 μCi [γ-32P]ATP (PerkinElmer) for 30 min at 30°C. The reactions were terminated by adding 6× SDS sample buffer followed by heating at 100°C for 5 min. The proteins were separated on a gradient SDS-polyacrylamide gel, and the incorporation of 32P was visualized by autoradiography.

Ionizing radiation. Ionizing radiation treatment was performed in the Department of Radiation Oncology in Ajou University Hospital using a Clinac 2100 C/D (Siemens AG).

Determination of cellular ROS levels. Cellular ROS level was determined by DCF-DA (Molecular Probes) staining. Cells were incubated with prewarmed medium containing 10 μM DCFH-DA for 10 min and washed with warm PBS twice. Cells were collected by trypsinization and resuspended with PBS. DCF-DA levels were analysed by FACS (Becton Dickinson) analysis.

Immunofluorescence staining and DNA damage foci counting. Cells were fixed with acetone/methanol (1:1 v/v) solution for 10 min at room temperature. The fixed cells were permeabilized with 0.5% Triton X-100 for 10 min and blocked with 3% BSA in PBS, followed by overnight incubation with appropriate primary antibodies at 4°C. Primary-antibody-incubated cells were washed three times with PBS and incubated with fluorescence-conjugated secondary antibody for 1 h. After washing the secondary antibodies from incubated cells, the nuclei were stained with DAPI (1:50 000, Molecular probes) for 10 min, followed by washing five times with 0.1% Triton X-100. Cells were mounted with Vectashield (Vector laboratories).

RT-PCR. Total RNA was isolated from cultured cells, using an RNasy RNA extraction Mini kit (Qiagen). One microgram of total RNA was used to synthesize cDNA with an RT-PCR reagent kit (Enzynomix). The resulting cDNA samples were quantified by PCR, using primers specific for retrotransposon L1 or albumin and visualized on an agarose gel. The following primer sets for human genes were used: GAPDH, (forward) 5’-ATGGGAAAGGTGAAGTCG-3’, (reverse) 5’-GGGG-TATTATGGCAAACTAATA-3’; retrotransposon L1 (VM164A) 5’-TGCTCCTCTAGTATGTTCCC-3’, (RB164K2) 5’-TGCTCCTCTCCCTCTATTGCC-3’; albumin, (forward) 5’-GGGATGTTTTGGT-3’, (reverse) 5’-CTGGTTACAAAGGTTCCTGT-3’.

Statistics. All bars represent the mean ± SD of determinants. Experiments were repeated at least three times and more than 200 cells were counted each time. Statistical analysis was performed by using the Student’s t-test.

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