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HLJ1 is a novel caspase-3 substrate and its expression enhances UV-induced apoptosis in non-small cell lung carcinoma

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ABSTRACT

Carcinogenesis is determined based on both cell proliferation and death rates. Recent studies demonstrate that heat shock proteins (HSPs) regulate apoptosis. HLJ1, a member of the DnaJ-like Hsp40 family, is a newly identified tumor suppressor protein closely related to relapse and survival in non-small cell lung cancer (NSCLC) patients. However, its role in apoptosis is currently unknown. In this study, NSCLC cell lines displaying varying HLJ1 expression levels were subjected to ultraviolet (UV) irradiation, followed by flow cytometry. Interestingly, the percentages of apoptotic cells in the seven cell lines examined were positively correlated with HLJ1 expression. Enforcing expression of HLJ1 in low-HLJ1 expressing highly invasive cells promoted UV-induced apoptosis through enhancing JNK and caspase-3 activation in NSCLC. Additionally, UV irradiation led to reduced levels of HLJ1 predominantly in apoptotic cells. The pan-caspase inhibitor, zVAD-fmk and caspase-3-specific inhibitor, DEVD-fmk, prevented UV-induced degradation of HLJ1 by the late stage of apoptosis. Further experiments revealed a non-typical caspase-3 cleavage site (MEID) at amino acid 125–128 of HLJ1. Our results collectively suggest that HLJ1 is a novel substrate of caspase-3 during the UV-induced apoptotic process.

INTRODUCTION

The past few decades have seen a dramatic increase in the number of publications on apoptosis. Apoptosis is an essential process during normal embryonic development, adult homeostasis and regulation of the immune system (1). Various cellular stress factors, including anti-cancer drugs, ionizing radiation and ultraviolet (UV) light, induce apoptosis and activation of signaling pathways (2–4). UV irradiation has multiple effects on cells, including DNA damage, and triggers expression of genes involved in DNA repair and apoptosis (5).

Heat shock proteins (HSPs) were initially identified in 1962 (6) as molecular chaperones induced by various stress conditions, including heat shock, exposure to radiation, heavy metals, ethanol, amino acid analogs, sodium arsenite and oxidative stress (7). HSPs are classified into six main groups according to molecular weight: Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and small HSP. Further studies in recent years indicate that HSPs regulate apoptosis, although the results to date are inconsistent. Hsp27 and Hsp70 are antiapoptotic proteins (8,9), whereas Hsp60 and Hsp10 promote the proteolytic maturation of caspase-3 (10). Moreover, Hsp105α prevents stress-induced apoptosis in neuronal PC12 cells (11), but enhances hydrogen peroxide-induced apoptosis in a mouse embryonic cell line (12). To date, Hsp40 has been characterized simply as a co-chaperone involved in the regulation of Hsp70 chaperone activity, but it is currently unclear whether this protein family is independently involved in the regulation of apoptosis (13). Additionally, the Hsp40 (DnaJ)–Hsp70 chaperone pair prevents against NO-induced apoptosis through

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The authors wish it to be known that, in their opinion, the last two authors should be regarded as joint First Authors.

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interactions with Bax and inhibition of translocation to mitochondria (14). However, it remains to be established whether HSPs are substrates of caspases.

Increasing attention is focused on DnaJ-like HSPs in tumor suppression analyses (15). HLJ1 is a DnaJ-like HSP belonging to the Hsp 40 family (16). In a previous study, we characterized HLJ1 as a novel tumor suppressor that inhibits cancer cell-cycle progression, proliferation, invasion and tumorigenesis, and is significantly correlated with prognosis in non-small cell lung carcinoma (NSCLC) patients (17). Moreover, HLJ1 is synergistically activated by the enhancer, AP-1, and promoter, YY1, through DNA bending (18,19). The mechanism of action of HLJ1 is of significant interest in the context of tumor suppression, but is yet to be fully explored. The objective of this study was to investigate the role of HLJ1 in apoptosis of lung cancer cells exposed to UV stress.

MATERIALS AND METHODS

Cell culture

Seven human NSCLC cell lines, CL1-0, CL1-1, CL1-5, CL1-5–F4, with different invasive capacities (20), NCI-H358 (ATCC CRL-5807), NCI-H1437 (ATCC CRL-5872), A549 (ATCC CCL-185) and one human cervical carcinoma cell line, HeLa, were maintained at 37°C in a humidified atmosphere of 5% CO_2. Cells were cultured in RPMI 1640 (GIBCO BRL, Grand Island, NY, USA) with 10% heat-inactivated fetal bovine serum (GIBCO BRL), and 1% penicillin and streptomycin (GIBCO BRL).

Construct preparation and transfection

To generate HLJ1 constructs, full-length HLJ1 cDNA was inserted into pcDNA3 (Invitrogen, Carlsbad, CA, USA), pEF6-V5/His (Invitrogen) and pQE-30 (Qiagen, Hilden, Germany), as described previously (17). For caspase-3 construct, full-length caspase-3 cDNA was inserted into pGEX-4T-1 vector containing GST tag (Amersham Pharmacia Biotech, Piscataway, NJ, USA). GST-tagged caspase-3 (C163S) (21), His-tagged HLJ1 (D128A) and V5-tagged HLJ1 (D128A) mutant constructs were produced with the QuikChange site-directed mutagenesis kit (Stratagene). All constructs were confirmed by DNA sequencing.

CL1-5 cells expressing low levels of HLJ1 were seeded in 6 cm dishes at 5×10^5 cells/dish and transfected with pcDNA3-HLJ1, pcDNA3 empty vector, pEF6-V5/His-HLJ1-D128A or pEF6-V5/His using Lipofectamine Reagent (Invitrogen), according to the manufacturer's protocol. After culturing in medium containing 400 μg/ml of geneticin (G418; GIBCO BRL) or 5 μg/ml of Blastidine (Invitrogen) for 2–3 weeks, single and mixed clones were isolated. For silencing HLJ1 expression, small-interfering RNA (siRNA) transient transfection was performed as previously described (17). The HLJ1-specific siRNA sequence and scrambled siRNA sequence were as follows, respectively, AACCCGGAAT GAGGAGAAGAA and GGACAATGAACACGAGGA AGA. The CL1-0 and HLJ1-2 were transfected with HLJ1-specific or scrambled siRNA using the RNAiFect Transfection Reagent (Qiagen). After culturing in the medium for 36 h, the cells were exposed to UV irradiation.

UV irradiation and apoptosis assay

Cells were seeded in 10 cm dishes at a concentration of 8×10^5 cells/dish. After a 24 h pre-incubation period, cells were exposed to 10, 20 or 50 J/m^2 of UV (254 nm) (BIO-LINK BLX-254, Vilber Lourmat, France), and allowed to recover for 3, 6, 9, 12, 24 or 48 h, depending on the experimental design. Aliquots of cells were pre-treated with 10 μg/ml cycloheximide (CHX; Calbiochem, CA, USA.), 20 μM zVAD-fmk (a broad caspase inhibitor, BD Biosciences, San Diego, CA, USA) or 30 μM DEVD-fmk (a caspase-3 specific inhibitor, BD Biosciences), and exposed to UV irradiation.

X-irradiation and drug treatment

X-irradiation was followed essentially as described previously (22). Cells were seeded in 75T flask at a concentration of 5×10^5 cells/flask. After pre-incubation period, cells were irradiated using 6 MV X-rays produced by a linear accelerator (SLi Precise 25001, ELEKTA) and allowed to recover for 72 h. X-ray doses ranged from 10 to 20 Gy. Paclitaxel (Bristol-Myers Squibb Co, Wallingford, CT) and staurosporine (Sigma, St. Louis, MO, USA) were prepared in sterile dimethylsulfoxid (DMSO; 99.8% pure; Sigma). The final concentration of DMSO in drug or mock control was <0.1%.

Western blot analysis

Western blot analysis was performed essentially as described previously (17). Cells were rinsed with PBS, and lysed in RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris–HCl and pH 7.5) containing protease inhibitors. Aliquots (30 μg proteins) of cell extracts were separated by SDS–PAGE, and transferred to PVDF membranes (Immobilon-P membrane; Millipore, Bedford, MA). The membranes were blocked with 5% skim milk in PBS containing 0.1% Tween-20 (PBST), followed by primary antibody in PBST/5% skim milk. The primary antibodies used for western blot analyses included monoclonal mouse anti-HLJ1 (made in-house), polyclonal goat anti-Hsc70 antibody (sc-1059, Santa Cruz Biotechnology, Santa Cruz, CA, USA), monoclonal mouse anti-Hsp70 antibody (sc-24, Santa Cruz Biotechnology), polyclonal rabbit anti-SAPK/JNK antibody (Cat. 9252, Cell Signaling Technology, Beverly, MA, USA), monoclonal mouse anti-phospho-SAPK/JNK (Thr183/Tyr185) antibody (Cat. 9255, Cell Signaling Technology), polyclonal rabbit anti-caspase-3 antibody (Cat. AB1899, Millipore), monoclonal mouse anti-caspase-9 antibody (Cat. 551246, BD Biosciences), polyclonal rabbit anti-PARP antibody (Cat. 5242, Cell Signaling Technology), and monoclonal mouse anti-β-tubulin (Cat. 05-661, Millipore) used as a loading control. Membranes were washed three times with PBST, followed by incubation with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) in PBST/5% skim

milk. Antibody–antigen complexes were detected using the Enhanced Chemiluminescence System (Amersham). All experiments were performed in triplicate.

Quantitative reverse transcription–PCR (RT–PCR)

The HLJ1 mRNA level was detected with SYBR Green real-time RT–PCR on an ABI Prism 7300 sequence detection system (Applied Biosystems). The TATA box-binding protein (TBP) was used as an internal control. The following primers were used: HLJ1 forward, 5′-CCAGCAGAC ATTGTTTTTATCATT-3′, HLJ1 reverse, 5′-CCATCCA GTGTTGGTACATTAATT-3′; TBP forward, 5′-CACG AACCACGGGACTGATT-3′ and TBP reverse, 5′-TTT TCTTGCTGCCAGTCTGGAC-3′. All experiments were performed in triplicate. The relative expression level of HLJ1, compared with that of TBP, was defined as $-\Delta CT = -(CT_{HLJ1} - CT_{TBP})$, whereas the relative expression ratio of HLJ1 between different treatments was calculated as $-\Delta\Delta CT = -(\Delta CT_{UV} - \Delta CT_{CTL})$. The ratio of UV: CTL or CHX+UV: CHX was estimated as $2^{-\Delta\Delta CT}$.

Flow cytometry

A flow cytometer was employed to determine the percentage of the sub-G1 cell population, indicative of UV-induced apoptosis. In brief, tested cells were seeded at a density of 5×10^5 cells/6 cm dish in medium with 10% FBS for 24 h, and exposed to 10, 20 or 50 J/m^2 of UV. After recovery for 6, 12, 24 or 48 h, each sample was washed with ice-cold PBS, harvested and fixed in 70% (v/v) ethanol for 2 h at −20°C. Cells were treated with RNase A, and stained with 25 μg/ml propidium iodide. Samples were analyzed using a Cytomics^{TM} FC500 flow cytometer (Beckman Coulter), according to the manufacturer's protocol. Cytometric data were analyzed with WinMDI 2.8 software. A minimum of 10 000 events were examined. Three independent experiments were performed.

In vitro cleavage of HLJ1 by recombinant caspase-3

Recombinant His-tagged HLJ1 was incubated with recombinant active caspase-3 (BD Biosciences) at 37°C for 2 h in 25 μl reaction buffer (50 mM HEPES, 0.1 M NaCl, 10% glycerol, pH7.5 and 10 mM DTT). The reaction was terminated by the addition of sample buffer, and analyzed by SDS–PAGE, followed by Coomassie blue staining. Proteins were identified with a Mass Spectrometer (MALDI-TOF, ABI Voyager-DE PRO, supported by Biotechnology Center, National Chung Hsing University) and N-terminal sequencing (ABI Procise 492, supported by Technology Commons, College of Life Science, National Taiwan University). In an inhibition experiment, recombinant caspase-3 was pre-incubated with 1 μM DEVD-fmk at room temperature for 15 min in the reaction buffer, and the reaction is initiated by the addition of recombinant His-tagged HLJ1.

Statistical analysis

Results are presented as mean ± SD. All experiments were performed in triplicate, and analyzed for significant differences using analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

RESULTS

Relationship between HLJ1 expression and UV-induced apoptosis in NSCLC cell lines

The endogenous HLJ1 protein levels in a variety of NSCLC cell lines are depicted in Figure 1A. Expression levels of HLJ1 were further digitized and normalized with internal control β-tubulin (Figure 1B). Highest HLJ1 expression was observed in CL1-0 and CL1-1, moderate expression in A549, H358 and H1437, and lowest expression in CL1-5 and CL1-5F4 cell lines. Interestingly, after UV irradiation at an intensity of 10 J/m^2 and cell recovery for 24 h, the apoptotic cell percentage was consistent with HLJ1 expression patterns (Figure 1C). The data show that the sub-G1 percentage of CL1-0 irradiated with UV increases 15.9-fold (from 1.42 ± 0.39% to 22.56 ± 1.08%). In contrast to the CL1-0 cell line displaying high HLJ1 protein expression, the sub-G1 percentage of CL1-5 cells expressing low HLJ1 levels barely increased (from 1.81 ± 0.97% to 2.36 ± 0.79%).

Augmentation of UV-induced apoptosis in HLJ1-expressing CL1-5 cells

To further establish the effects of HLJ1 on UV irradiation-induced apoptosis, HLJ1-expressing or empty vector was introduced into CL1-5 cells expressing low levels of HLJ1. The morphological properties of CL1-5 cells transfected with HLJ1 (HLJ1-1 and HLJ1-2) were altered, compared with those of mock transfectants, mock-1 and mock-2 (Figure 2A). Upon HLJ1 transfection, CL1-5 cells displayed a less elongated cell shape. Cells lost their fibroblast-like appearance and assumed epithelial cell morphology, without a bipolar or spindle shape, and flattened onto plates, similar to CL1-0 cells.

Next, we examined the effects of HLJ1 overexpression on UV-induced apoptosis in the CL1-5 cell line. Clones stably expressing HLJ1 were identified by western blot, and subjected to the apoptotic assay (Figure 2B). Cell lines, including CL1-5, mock controls and HLJ1 transfectants, were exposed to 50 J/m^2 of UV, and allowed to recover for 6, 12 or 48 h before analysis of the percentage of apoptosis with flow cytometry. Induction of HLJ1 expression led to a significant elevation in the apoptotic cell percentage by ∼2–3-fold, compared to that of cells containing the empty vector (Figure 2C). On the contrary, silencing HLJ1 with HLJ1-specific siRNA would cause a marked decrease of apoptotic cell percentage, compared to the scrambled control (Figure 2D).

Induction of HLJ1 expression leads to enhanced activation of JNK and caspase-3 after UV irradiation

JNK pathways are activated by UV stress, resulting in the caspase cascade and initiation of apoptotic processes, and

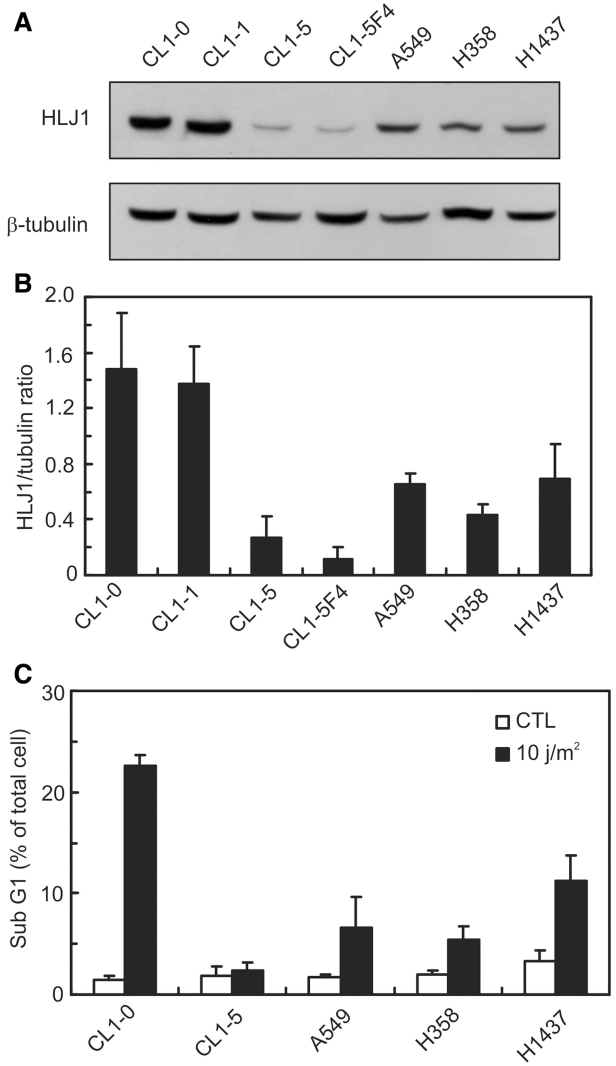

Figure 1. HLJ1 expression and UV-induced apoptosis in NSCLC cell lines. (**A**) Expression of HLJ1 in different NSCLC cell lines. HLJ1 was detected by western blot analysis with β-tubulin as an internal control. (**B**) Relative expression of HLJ1 was calculated by normalizing against the corresponding densities of the internal control. (**C**) UV irradiation induces cell apoptosis. NSCLC cell lines were exposed to 10 J/m^2 of UV and allowed to recover for 12 h. Apoptotic cells were determined as the sub-G1 percentage using flow cytometry. CTL, no treatment control. Data are presented as mean ± SD of three experiments.

are implicated as key regulators of stress-induced apoptosis in a variety of cell types (4,23). To determine whether HLJ1 is involved in JNK signaling pathways, we examined the phosphorylation of JNK in CL1-5 cells exposed to UV stress. The results illustrated in Figure 3A provide evidence that UV stress induces marked JNK activation within 15 min in HLJ1 transfectants, compared with mock control cells. It appears that enforced expression of HLJ1 enhances the UV stress-induced activation of JNK. Accordingly, we further assayed caspase-3 and -9 activities in UV-exposed cells. Both caspase activities remained unchanged for 24 h in mock control cells, but increased markedly in HLJ1-transfected cell clones within 12 h (Figure 3B). PARP is an important substrate of caspase-3. Interestingly, cleavage of PARP under UV stress conditions was markedly increased in HLJ1 transfectants, compared to mock controls (Figure 3B), consistent with the increased caspase-3 activity.

Reduction of HLJ1 in UV-exposed cells

To determine the dynamic relationship between HLJ1 and cell death, dose-effect and time-course responses of HLJ1 expression and apoptosis to UV exposure were analyzed. CL1-0 cells were exposed to: (i) UV at intensities of 10, 20 or 50 J/m^2, followed by recovery for 6 h; and (ii) 10 J/m^2 of UV and recovery over a period of 3–24 h. Western blot analyses disclose a dose-dependent decrease in the HLJ1 level, compared with non-treated control, at 10, 20 or 50 J/m^2 UV exposure (Figure 4A). However, Hsc70 and Hsp70 levels remained unaffected under all assay conditions. The amounts of HLJ1 in CL1-0 recovered at 3, 6, 9, 12 and 24 h after UV exposure were reduced to 1-, 0.75-, 0.48-, 0.35- and 0.35-fold, compared with control, respectively (Figure 4B). Again, no alterations in Hsc70 were observed at all assay time-points. The decrease in HLJ1 was accompanied by increased caspase-3 activity and cleavage of PARP. In addition, decreased HLJ1 levels were observed in transfectants derived from CL1-5, HLJ1-1 and HLJ1-2, following irradiation with 50 J/m^2 UV and recovery for 12 h (Figure 4C). This result is similar to that obtained in Figure 2D.

HLJ1 is cleaved by caspase in apoptotic cells

To establish the mechanisms underlying HLJ1 reduction, further experiments were performed with regard to inhibition of transcription and acceleration of protein degradation. Prior to exposure to UV, CL1-0 cells were pre-incubated with CHX to inhibit *de novo* protein synthesis. Western blot analyses showed that UV exposure led to a decrease in the HLJ1 level by 28.7%, compared with non-treated control. However, upon pre-treatment with CHX, a reduction of 70.3% was observed (Figure 5A, left). Furthermore, the addition of CHX to cell culture did not affect the reduction level of HLJ1 mRNA, indicating that degradation occurs at the protein level (Figure 5A, right).

To determine the group of cells constituting the main source of HLJ1 reduction after UV irradiation, total, detached and adherent cells were harvested and subjected to flow cytometry. The sub-G1 percentage of total cells exposed to UV irradiation was up to 44.97%, while that of detached cells was 61.69% (Figure 5B). In contrast, the sub-G1 percentage of adherent cells was only 7.11%, similar to control cells not exposed to UV. Annexin-V staining disclosed similar results (data not shown). Furthermore, in CL1-0 and HeLa cultures, the amount of HLJ1 was obviously decreased in the total and detached cell populations, particularly in the latter almost completely depleted of HLJ1 (Figure 5C). Compared with control cells, the HLJ1 protein level in adherent cells was only slightly decreased after UV exposure.

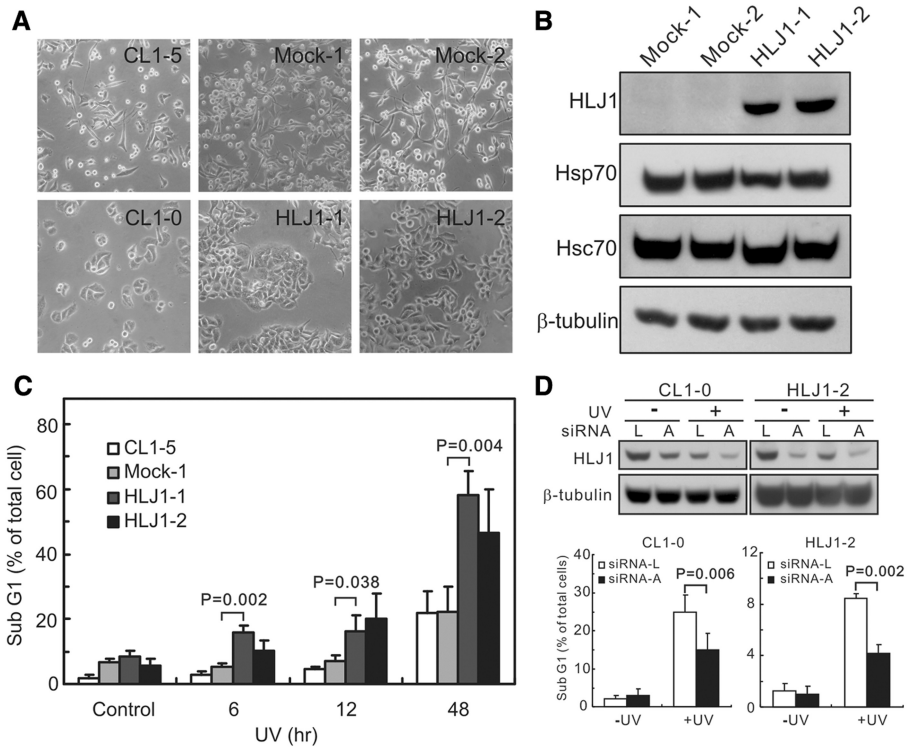

Figure 2. UV-induced apoptosis in HLJ1-expressing CL1-5 cells. (**A**) HLJ1 induces morphological changes in lung cancer cells. Cells were plated in dishes for 24 h, and photographed using a microscope (optical magnification: $\times 200$). (**B**) Generation of lung cancer cell lines constitutively expressing HLJ1. The HLJ1 protein level was determined by western blot analysis. Levels of ectopically expressed HLJ1 in HLJ1-1 and HLJ1-2 cell lines were higher than those of endogenous HLJ1 in the CL1-5 cell lines, mock-1 and mock-2. (**C**) HLJ1 augments cell apoptosis induced by UV irradiation. CL1-5, mock and HLJ1 transfectants were exposed to 50 J/m^2 of UV irradiation, and allowed to recover for the indicated times (6, 12 and 48 h). The percentages of apoptotic cells were quantified by flow cytometry, and presented as mean ± S.D. of three experiments. (**D**) Reduction of HLJ1 diminishes cell apoptosis induced by UV exposure. L, scrambled control; A, HLJ1 silence. The percentages of apoptotic cells are presented as mean ± S.D. of three experiments.

In this model of UV-induced degradation of HLJ1, we further investigated the effects of the pan-caspase inhibitor, zVAD-fmk and the caspase-3-specific inhibitor, DEVD-fmk, on UV-induced apoptosis. CL1-0 cells were pre-treated with the inhibitors and subjected to UV treatment. Both zVAD-fmk and DEVD-fmk significantly inhibited UV-induced cell apoptosis in flow cytometry analysis ($\alpha = 0.05$, $P = 0.0002$ and $P = 0.001$, respectively) (Figure 5D). Moreover, western blot analysis revealed that zVAD-fmk and DEVD-fmk blocked the degradation of HLJ1 as well as the caspase-3 substrate, PARP (Figure 5E). Based on the data, we propose that HLJ1 is cleaved by caspases, in particular, caspase-3, during apoptosis induced by UV irradiation.

HLJ1 is a novel substrate of caspase-3 cleaved at Asp128

The lung cancer cell line, CL1-5, was transiently transfected with V5-tagged HLJ1, irradiated with 50 J/m^2 of UV, and allowed to recover for 24 h. A distinct band was observed at ~31 kDa (Figure 6A). To determine the specific cleavage site of caspase-3 in HLJ1, bacterially expressed His-tagged HLJ1 was purified and treated with various amounts of active recombinant caspase-3. HLJ1 was cleaved by caspase-3 in an enzyme concentration-dependent manner to produce two fragments with molecular weights of about 24 and 15 kDa, respectively (Figure 6B). The addition of DEVD-fmk to the reaction prevented the degradation of HLJ1. The two bands were confirmed by MALDI-TOF mass spectrometry. Furthermore, N-terminal amino acid sequencing of 24 kDa fragment revealed that caspase-3 cleaves HLJ1 at D^{128} of MEID^{128}G (Met–Glu–Ile–Asp^{128}–Gly) (Figure 6C). To confirm the cleavage site, an *in vitro* cleavage assay was performed using the mutant HLJ1 (D128A) and inactive caspase-3 (C163S). In contrast to wild-type HLJ1, HLJ1-D128A was not cleaved into the 24 and 15 kDa products by active caspase-3. Additionally, HLJ1 was not cleaved by an inactive form of caspase-3. BSA was employed as a negative control (Figure 6D).

Cleavage of HLJ1 is required for apoptosis

To determine the relationship between HLJ1 cleavage and UV-induced apoptosis, V5-tagged HLJ1 and mutant HLJ1 (D128A) stable transfectants were exposed to UV irradiation, followed by flow cytometry. The results showed that the sub-G1 percentage of V5-tagged HLJ1-transfected cells was significantly increased, as compared with mock cells ($\alpha = 0.05$, $P = 0.0001$) (Figure 7A). Interestingly, mutant HLJ1-transfected cells showed a marked decrease of apoptosis, compared to HLJ1 transfectants ($\alpha = 0.05$, $P = 0.0001$). We further

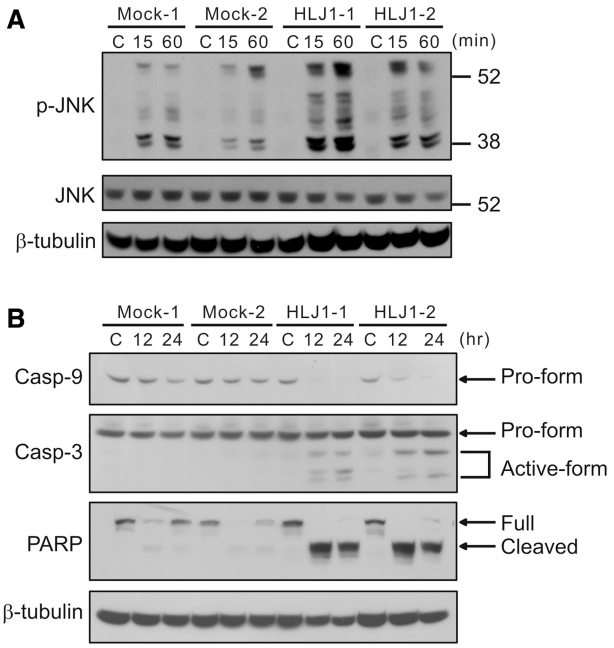

Figure 3. Effect of HLJ1 on activation of JNK and caspase-3 after UV irradiation. (**A**) Ectopic expression of HLJ1 enhances JNK activity after UV irradiation. Mock (mock-1 and mock-2) and HLJ1 transfectants (HLJ1-1 and HLJ1-2) were irradiated with 50 J/m^2 of UV, followed by recovery for 15 or 60 min. Activated and total JNKs were detected by western blotting with antibodies specific for JNK and p-JNK, respectively. (**B**) Enforced expression of HLJ1 enhances caspase-3 and -9 activities. Mock and HLJ1 transfectants were exposed to 50 J/m^2 of UV, and allowed to recover for 12 or 24 h. Cell extracts were analyzed for caspase-3, -9 and PARP by western blotting with anti-caspase-3, anti-caspase-9 and anti-PARP antibodies. β-tubulin was used as a control for protein loading.

examined the phosphorylation of JNK and the activity of caspase in mutant HLJ1 transfectant exposed to UV stress. Mutant HLJ1 (D128A) inhibited JNK activation by reducing phosphorylation of JNK but had no effect on JNK protein level (Figure 7B). Enforced expression of HLJ1 increased the activation of caspase-9 and the cleavage form of PARP in UV-exposed cells, while mutant HLJ1 recovered the phenomenon (Figure 7C). Moreover, HLJ1 mutation blocked the reduction of HLJ1 caused by UV stress. All of these indicators showed that HLJ1 is cleaved at Asp128 *in vivo* and HLJ1 cleavage is required for UV-induced apoptosis.

Reduction of HLJ1 is appeared in other stress-induced apoptosis

To investigate the effect of HLJ1 on other stress-induced apoptosis, ionizing irradiation and drugs were employed to treat CL1-0, CL1-5 and stable HLJ1-transfected cells. The results are illustrated in the Supplementary Material. The sub-G1 percentage of cells was measured after 10, 15 or 20 Gy X-irradiation and recovery for 72 h. Just like UV-induced apoptosis, the apoptotic cell percentage caused by X-irradiation in CL1-0 was increased more markedly than in CL1-5 ($P = 0.079$ at 10 Gy; $P = 0.014$ at 15 Gy; $P = 0.003$ at 20 Gy) (Supplementary Figure S1A, left). And HLJ1 overexpression in CL1-5 cells would enhance the sub-G1 percentage as compared with mock at the indicated irradiation doses, from 22.39%, 46.65% and 56.02% to 30.25%, 52.70% and 67.37%, respectively (Supplementary Figure S1A, right). However,

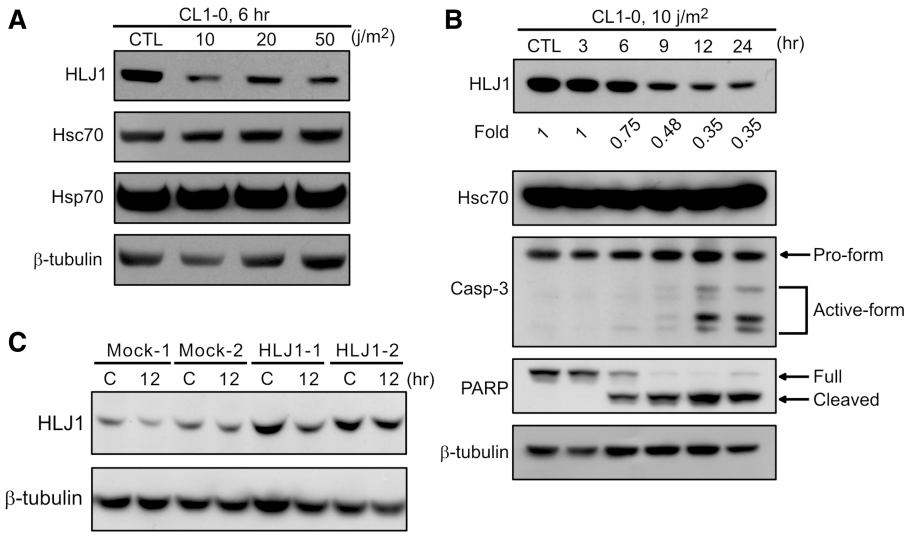

Figure 4. HLJ1 reduction by UV irradiation. (**A**) HLJ1 is decreased following UV exposure in a dose-dependent manner. CL1-0 cells were irradiated with 10, 20 or 50 J/m^2 of UV, and allowed to recover for 6 h. Expression of HLJ1, Hsc70 and Hsp70 was detected by western blot analysis. (**B**) HLJ1 is decreased after UV exposure in a time-dependent manner. CL1-0 cells were irradiated with 10 J/m^2 of UV, and allowed to recover for 3, 6, 9, 12 or 24 h. Cell extracts were analyzed by western blot with anti-HLJ1, anti-Hsc70, anti-PARP and anti-caspase-3 antibodies. (**C**) Ectopically expressed HLJ1 is decreased after UV exposure. Mock- and HLJ1-transfected cells derived from CL1-5 were exposed to 50 J/m^2 of UV irradiation, followed by recovery for 12 h. HLJ1 was detected by western blot analysis using an anti-HLJ1 antibody. β-tubulin was used as the internal control.

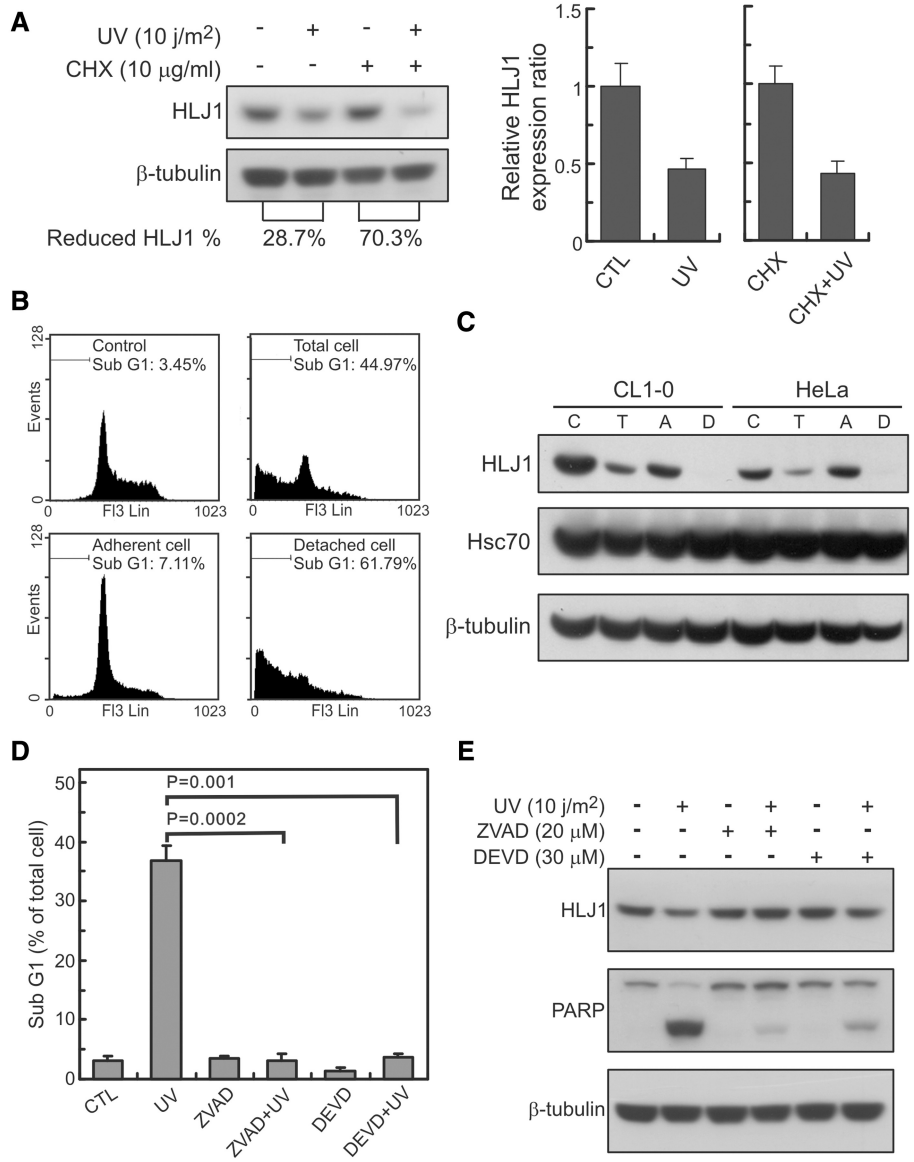

Figure 5. Caspase-dependent HLJ1 reduction in apoptotic cells. (**A**) Decreased expression of HLJ1 was pronounced upon inhibition of protein synthesis. CL1-0 cells were pre-incubated with 10 μg/ml of CHX for an hour, and exposed to 10 J/m^2 of UV. After recovery for 6 h, HLJ1 expression was analyzed by western blot using anti-HLJ1 antibody (left) and real-time RT–PCR (right). (**B**) The majority of detached cells induced by UV irradiation were apoptotic. CL1-0 cells were irradiated with 10 J/m^2 of UV, and allowed to recover for 24 h. Based on adherence, total cells including detached and adherent, adherent cells and detached cells, were harvested. The DNA content of cells was analyzed by flow cytometry, as described in 'Materials and Methods' section. (**C**) HLJ1 was absent in detached cells. CL1-0 and HeLa cells were treated with 10 J/m^2 of UV irradiation, and allowed to recover for 24 h. Total cells (T), adherent cells (A) and detached cells (D) were collected for western blot analysis with anti-HLJ1 and anti-Hsc70 antibodies. β-Tubulin was applied as the internal control. Results are representative of three independent experiments. (**D**) Caspase inhibitors attenuated UV-induced cell apoptosis. CL1-0 cells were pre-incubated for 3 h with 20 μM zVAD-fmk or 30 μM DEVD-fmk, followed by exposure to 10 J/m^2 of UV irradiation. After recovery for 6 h, the apoptotic cell content was determined based on the sub-G1 proportion using a flow cytometer. Data are presented as mean ± S.D. (n = 3). (**E**) Caspase inhibitors blocked UV-induced HLJ1 degradation. Western blots were performed with antibodies against HLJ1, PARP or β-tubulin. PARP is a caspase-3 substrate, while β-tubulin is the internal control.

the enforced expression of HLJ1 did not result in the obvious sensitivity to the drugs used in this study (data not shown). We next investigated if HLJ1 is also cleaved in apoptotic cells caused by X-irradiation or drugs including paclitaxel and staurosporine. Our results demonstrated a dose-dependent decrease of HLJ1 protein in CL1-0 and CL1-5 exposed to X-irradiation (Supplementary Figure S1B). The result also revealed that HLJ1 could be cleaved to yield the cleaved form in stable transfectant treated with X-ray irradiation, paclitaxel or staurosporine, while mutant HLJ1 (D128A) blocked the processing (Supplementary Figure S1C and D). In addition, PARP was cleaved in parallel with wild-type HLJ1; however, the cleavage was reduced with mutant HLJ1 (D128A) in all assays, suggesting that the cleavage of HLJ1 is required for caspase-3 activity.

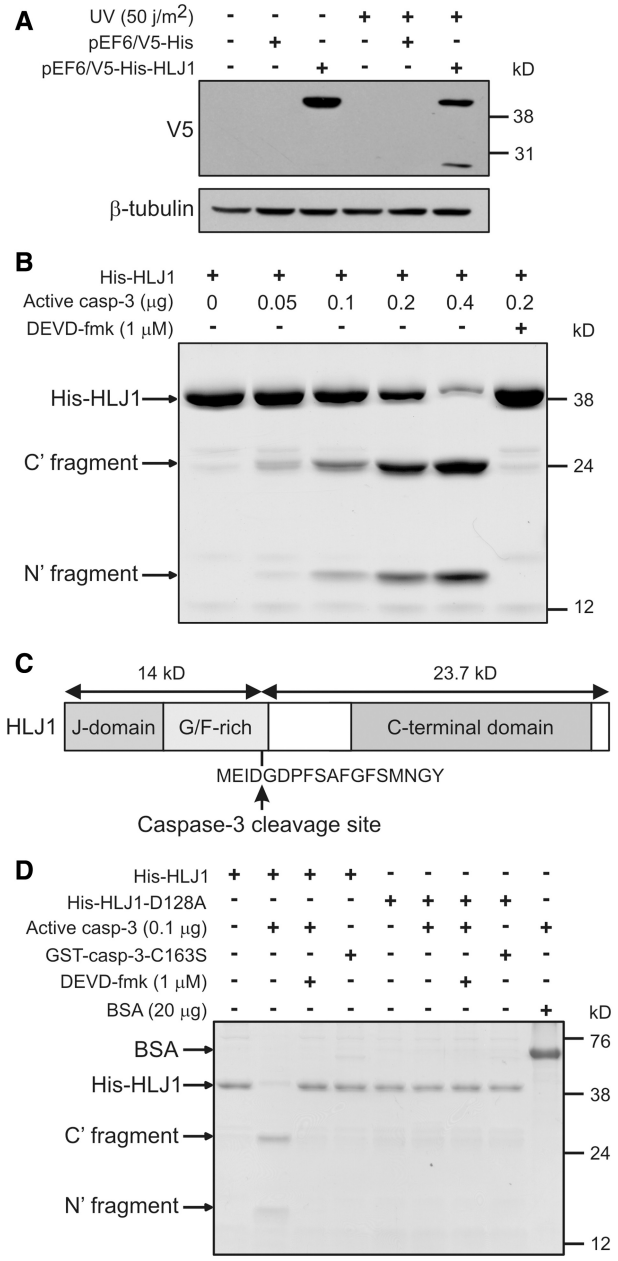

Figure 6. Caspase-3 cleavage site of HLJ1. (**A**) *In vivo* cleavage of HLJ1 by exposure to UV stress. CL1-5 cells were transiently transfected with V5-tagged HLJ1 and exposed to 50 J/m^2 of UV irradiation. After recovery for 24 h, induced HLJ1 expression was detected by western blot analysis with an anti-V5 tag antibody. (**B**) *In vitro* cleavage of HLJ1 by recombinant caspase-3. The purified His-tagged HLJ1 was incubated with a series of concentrations of active caspase-3 at 37°C for 2 h, and subjected to SDS–PAGE followed by Coomassie blue staining. (**C**) Illustration of caspase-3-mediated cleavage of HLJ1. The cleaved 24 kDa HLJ1 fragment was subjected to N-terminal amino acid sequencing. The cleavage site is marked with an arrow at position 128 (Asp) in the G/F-rich domain. (**D**) Effects of HLJ1-D128A and caspase-3-C163S in an *in vitro* cleavage assay. Purified His-tagged HLJ1 or HLJ1-D128A were incubated with active caspase-3 or GST-caspase-3-C163S at 37°C for 2 h, and analyzed using SDS–PAGE, followed by Coomassie blue staining. BSA was employed as a negative control.

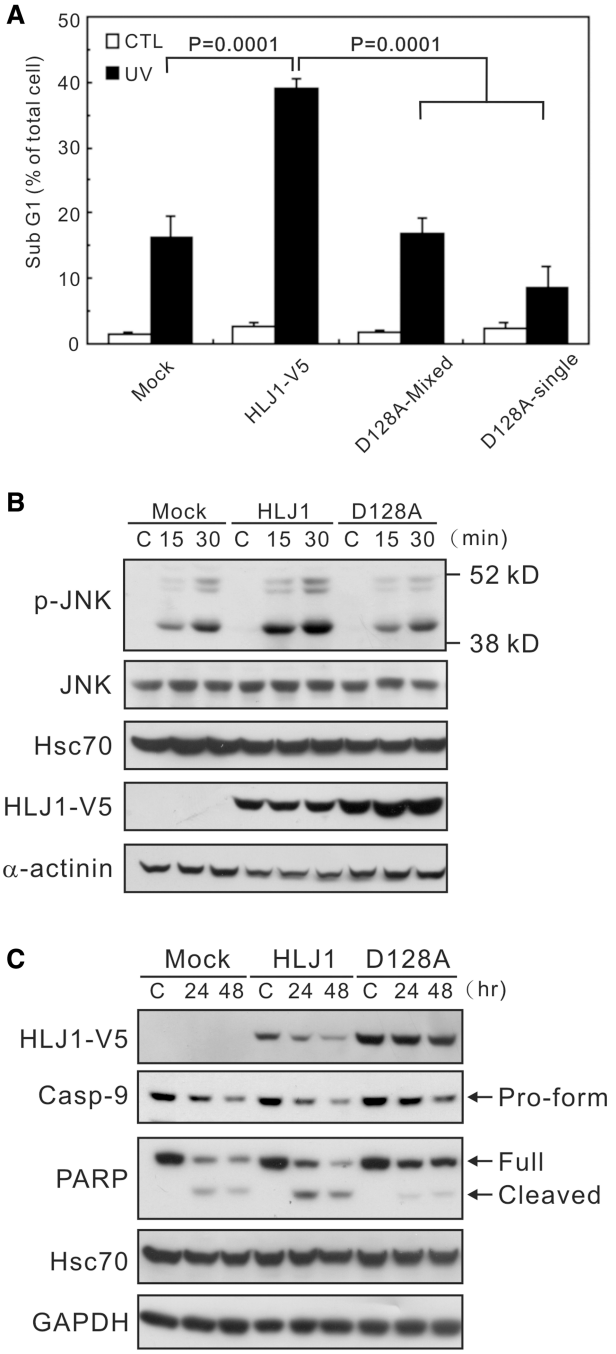

Figure 7. Effect of HLJ1 cleavage on UV-induced apoptosis. (**A**) HLJ1-D128A mutant is resistant to UV-induced apoptosis. After UV irradiation, the sub-G1 percentages of vector-transfected control cells (Mock), HLJ1-transfected cells (HLJ1) and mutant HLJ1-transfected cells (D128A-mixed clone and D128A-single clone) were detected by flow cytometry. Data are presented as mean ± S.D. of three independent experiments. (**B**) UV-induced JNK activation is defective in mutant HLJ1 transfectant. Wild-type HLJ1 and mutant HLJ1 (D128A) transfectants were irradiated by UV light followed by incubation at 37°C for 15 or 30 min. JNK activity was detected by western blotting with an anti-p-JNK antibody. α-Actinin served as the loading control. The expression of V5-tagged HLJ1 in transfectant was detected with an anti-V5 tag antibody. (**C**) Rescue of the caspase pathway by expression of HLJ1-D128A mutant protein. Mock, HLJ1 and mutant HLJ1 transfectants were exposed to 50 J/m^2 of UV, and recovered for 24 and 48 h. Cell extracts were analyzed for V5-tagged HLJ1, caspase-9 and PARP by western blotting with the indicated antibodies. GAPDH was used as a control for protein loading.

DISCUSSION

Evasion of apoptosis is a hallmark of most cancers. Thus, it is important to identify genes that promote apoptosis in cancer cells, either under normal or stress conditions, such as radiotherapy and chemotherapy. Several recent studies show that HSPs play important cytoprotective roles, and are involved in regulation of the apoptosis pathway (24). HLJ1, a member of the Hsp 40 family, is a newly identified tumor suppressor closely related to tumorigenesis as well as a predictor of relapse and survival in NSCLC patients (17). However, the role of HLJ1 in apoptosis is currently unknown. In this study, we demonstrate that HLJ1 promotes UV-induced apoptosis through enhancing JNK and caspase-3 activation in NSCLC. Moreover, HLJ1 is a novel target of caspase-3 that is degraded at the late stage of apoptosis. A non-typical caspase-3 cleavage site (MEID) located at positions 125–128 of HLJ1 has been identified.

While it is implied that HSPs regulate apoptosis, diverse results have been obtained to date. For instance, Hsp27 prevents cell death from gamma irradiation in Jurkat–Hsp27 cells (25) and inhibits Bax-mediated apoptosis via a PI3K-dependent pathway in renal epithelial cells (26). Hsp70 acts against apoptosis induced by a variety of stress conditions through suppression of JNK activation, preventing recruitment of procaspase-9 to the Apaf-1 apoptosome and inhibiting caspase-3 activation (27). In contrast, Hsp60 and Hsp90 play both anti- and pro-apoptotic roles. Release of mitochondrial Hsp60/Hsp10 stimulates the activation of caspase-3 in HeLa and Jurkat cells, and Hsp90 promotes the rate of TNF-induced apoptosis (10,28). Other studies show that overexpression of Hsp60 enhances the survival rate in cardiac myocytes during ischemia injury (29), and Hsp90 directly binds Apaf-1 to inhibit the activation of caspase-9 (30). On the other hand, oxidative stress-induced apoptosis was enhanced by Hsp105α in mouse embryonic F9 cell lines, but reduced in neuronal PC12 cells (11,12). To our knowledge, this is the first report to show that HLJ1, a Hsp40 protein, acts as an enhancer of apoptosis by increasing JNK and caspase-3 activities in the NSCLC cell line.

JNK, also designated stress-induced kinase, is part of a family of mitogen-activated protein (MAP) kinases, including ERK and p38 kinase (4). Stimulation of the JNK signaling pathway induced by external stress factors, such as UV irradiation, leads to apoptosis (4). The JNK pathway is involved in activation of caspases and initiation of the apoptotic process in a variety of cell types (31,32). Our results indicate that the increase in JNK activity is more marked in HLJ1-overexpressing than mock control cells (Figure 3A). Thus, HLJ1 may promote UV stress-induced apoptosis through enhancing JNK activation in a CL1-5 cell line. The apoptosis pathway involves activation of caspases, which are stimulated in a proteolytic cascade to cleave specific substrates, such as PARP (33). Caspase activation is initiated through two major apoptotic mechanisms, specifically, the mitochondrial death and receptor-mediated cell death pathways. The mitochondrial death pathway follows the release of cytochrome c from mitochondria and activates caspase-9, which, in turn, activates downstream effector caspases, such as caspase-3 (34). A previous report additionally showed that JNK promotes caspase activation through cytochrome c release and apoptosome activation induced by UV stress (35). Here, we demonstrate that enforced expression of HLJ1 enhances PARP cleavage and caspase-3 activity in CL1-5 cells irradiated with UV, ultimately leading to increased apoptosis.

In general, HSPs display basal expression levels in non-stressed cells. Earlier studies indicate that expression levels of these proteins are strongly elevated under stress conditions (36). In contrast, our data show that the amount of HLJ1 is reduced in UV-exposed cells, and almost completely depleted by the late apoptotic stage. During apoptotic progression, caspases cleave specific substrates and mediate biochemical and morphological changes, such as cell shrinkage and membrane blebbing (37). Recently, several classes of substrates that are cleaved by caspases during apoptosis have been identified, including proteins involved in DNA metabolism and repair, signal transduction pathways, and regulation of cell cycle and proliferation, as well as those playing a direct role in apoptosis following cleavage (38). An article published in 1998 indicates that Hsp90 is a candidate substrate of apoptosis-associated proteolysis (39). However, there is no direct evidence confirming that HSPs are substrates of caspases. Surprisingly, data from our UV irradiation-induced apoptotic model confirm that HLJ1 is a substrate of caspase-3, and its reduction is attributed to cleavage at position 128 (aspartic acid). Caspase-3 usually cleaves substrates with a consensus motif of DXXD (P4–P3–P2–P1) (38) and the P1 position has absolute selectivity for Asp. Nevertheless, several caspase-3 substrates, such as STAT1 (40) and HAX (21) lack a typical DXXD motif. Additionally, a recent review reported a preference for glycine, alanine, threonine, serine and asparagine in P1′, and the invariant preference of the P3 position is glutamic acid for all mammalian caspases (41,42). While the caspase-3 cleavage motif, MEID, in the HLJ1 protein is non-typical, the amino acids at positions P1 and P3 are consistent with the above findings. However, this novel motif in HLJ1 is unique, and distinct to previously identified cleavage sites.

In addition to UV stress, the other stresses that can induce apoptosis to cancer cells by various mechanisms, such as X-irradiation, paclitaxel and staurosporine, are included in this study to investigate the role of HLJ1 in apoptosis and the cleavage of HLJ1 caused by caspase-3. X-irradiation, an ionizing radiation, induces both single- and double-strand breaks and causes a lethal effect. Paclitaxel, a mitotic inhibitor, interferes with the breakdown of microtubules during cell division and induces cell arrest in the *G2*/*M* phase. Staurosporine is a potent inhibitor of phospholipid/Ca^{2+}-dependent protein kinase (protein kinase C; PKC) and widely employed as an inducer of apoptosis in many mammalian cell types (43). We found that overexpression of HLJ1 in CL1-5 cells also enhances X-irradiation-induced apoptosis, such as UV-induced apoptosis. These data indicate that HLJ1 is associated with radiosensitivity. The cleaved HLJ1 was

also observed when CL1-0, CL1-5 and HLJ1-transfected cells were treated with X-irradiation or drugs, but not observed in mutant HLJ1-transfected cells. Moreover, our results showed that overexpression of HLJ1 enhances PARP cleavage caused by caspase-3 activity in CL1-5 exposed to UV-irradiation, X-irradiation or drugs, even though HLJ1 overexpression has no apparent effect on drug-induced apoptosis. In particular, the expression of mutant HLJ1 (D128A) decreased the cleavage of PARP in all assays. Taken together, these results implied that HLJ1 could enhance the apoptosis at or upstream of caspase-3 activation and the cleavage of HLJ1 might also enhance the activation of caspase-3.

HLJ1, also designated DNAJB4, contains three distinct domains (13) (Figure 6C): (i) the J domain (amino acids 1-67) near the N-terminus that mediates interactions with Hsp70 and regulates ATPase activity of Hsp70; (ii) a glycine and phenylalanine (G/F)-rich region (positions 68–141) that is disordered and possibly acts as a flexible linker; and (iii) the C-terminal domain (positions 200–321) forming a β-sheet structure involved in substrate binding and presentation. A recently published prediction model of HLJ1–Hsp70 interactions implies that HLJ1 binds the ATPase domain of Hsp70 through its J domain and the C termini of both HSPs interact together (13). Our results indicate that HLJ1 is cleaved by caspase-3 at D^{128} of $MEID^{128}G$, which is located at the terminus of the G/F-rich region possibly responsible for flexibility. Therefore, it is reasonable to speculate that caspase-cleaved HLJ1 loses the ability to interact with both the ATPase and substrate-binding domains of Hsp70, leading to loss of function, despite no decrease in the Hsp70 level during apoptosis.

In conclusion, HLJ1 promotes the sensitivity of cancer cells to UV stress-induced apoptosis through enhancing JNK activation and caspase activity. Moreover, the HLJ1 protein is cleaved by caspase-3, followed by protein degradation during the apoptotic process. HLJ1 appears to play an important role in apoptosis. However, further studies are necessary to determine the underlying mechanism of HLJ1 in UV-induced apoptosis and the effects of HLJ1 reduction during the late apoptotic process. A therapeutic strategy based on induced expression of HLJ1 may be developed in the future, with a view to synergistically elevate the effects of radiotherapy and prolonge patient survival.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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