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The novel gene LRP15 is regulated by DNA methylation and confers increased efficiency of DNA repair of ultraviolet-induced DNA damage

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LRP15 is a novel gene cloned from lymphocytic cells, and its function is still unknown. Bioinformatic data showed that LRP15 might be regulated by DNA methylation and had an important role in DNA repair. In this study, we investigate whether the expression of LRP15 is regulated by DNA methylation, and whether overexpression of LRP15 increases efficiency of DNA repair of UV-induced DNA damage in HeLa cells. The results showed (1) the promoter of LRP15 was hypermethylated in HeLa cells, resulting a silence of its expression. Gene expression was restored by a demethylating agent, 5-aza-2'-deoxycytidine, but not by a histone deacetylase inhibitor, trichostatin A; (2) overexpression of LRP15 inhibited HeLa cell proliferation, and the numbers of cells in the G2/M phase of the cell cycle in cells transfected with LRP15 increased about 10% compared with controls; (3) cyclin B1 level was much lower in cells overexpressing LRP15 than in control cells; and (4) after exposure to UV radiation, the LRP15-positive cells showed shorter comet tails compared with the LRP15-negative cells. From these results we conclude that the expression of LRP15 is controlled by methylation in its promoter in HeLa cells, and LRP15 confers resistance to UV damage and accelerates the DNA repair rate. [BMB reports 2008; 41(3): 230-235]

INTRODUCTION

LRP15 is a novel gene which was cloned from human lymphocyte cells by our group in 2001 using restriction length genomic scanning (RLGS), and then the cDNA was isolated using the rapid amplification of cDNA end (RACE) technique (GenBank Accession No. AF396933) (1). It's 1718 bp full length cDNA contains an open reading frame encoding a protein of 259 amino acid. A Genbank search did not reveal a known protein with structural similarity to LRP15, and the detailed function of this gene is not exactly known (2).

Based on a computer-aided SAGE (serial analysis of gene expression) pattern analysis, we found that LRP15 mRNA was highly expressed in the normal brain, kidney, lung, liver, testis and adrenal gland, while it was lowly expressed in some cancer cells such as HeLa, MCF-7, HL-60 and K562. It was recognized that gene expression is controlled in normal tissues at the transcriptional level, and aberrant promoter methylation might be the mechanism responsible for the altered expression of tumor associated genes (3-5). Aberrant promoter methylation is considered a fundamental process in developing cancers and has recently received considerable interest as a rapid non-invasive molecular screening tool for the early detection of tumor cells in a range of bodily fluids and biopsy specimens (6, 7). Our recent studies have likewise suggested that methylation of LRP15 promoter DNA at areas of CpG islands may result in aberrant LRP15 methylation in human acute myeloblastic leukemia (3). It stimulated us to ask whether the expression of LRP15 was regulated by promoter methylation of this gene.

Bioinformatics data showed LRP15 protein contained a structural motif (234 residues in length), which was a signal of neucleus localization. These results of bioinformatic analysis were verified by experiments with enhanced green fluorescence protein (EGFP) vector and laser confocal microscopy (2, 5). The data also showed the protein contained a leucine rich repeat N-terminal domain seemingly important for such diverse molecular processes as signal transduction, cell development, DNA repair and RNA processing. Based on these studies, we assumed that whether LRP15 gene played a role in DNA repair.

In this study, the methylation status of a high-density CpG island promoter region of LRP15 was described. We explored whether this region was aberrantly methylated in human cervical cancer cell line (HeLa) and whether a correlation existed between methylation and gene expression. The expression vector pcDNA3.1 containing the entire coding region of LRP15 gene was transfected into HeLa cells, and the expression levels of p53, $p21^{WAF1/CIP1}$ and cyclin B1 proteins, the cell cycle and the effect of LRP15 gene expression on the DNA damage and repair were observed.

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RESULTS

Expression and methylation status of LRP15 gene

RT-PCR showed that the expression of LRP15 mRNA was detected in COS7 cells, while not in HeLa cells (Fig. 1A). GPI was used as a control for RNA integrity. MS-PCR showed that complete methylation of the promoter region was seen in HeLa cells, while complete unmethylation in COS7 cells (Fig. 1B). To confirm our MS-PCR findings, we performed bisulfite genomic sequencing of the sodium bisulfite modified DNA. The cytosines in the non-CpG sites were converted to thymidine but the cytosines associated with CpG islands remained unmodified after bisulfite treatment thereby demonstrating complete methylation (Fig. 1C).

5-Aza-CdR and TSA treatment of HeLa cells

The expression of this gene in HeLa cells was so low that it could not be reliably detected using RT-PCR. However, by the 5-aza-CdR (with or without TSA) treatment, as shown in Fig. 2A, both demethylation of the promoter CpG island and activation of transcription of LRP15 gene were achieved. The minimum concentration of 5-aza-CdR which could induce mRNA expression was 1.0 μmol/l, and the demethylation of the promoter CpG islands was complete (Fig. 2A). Despite of the fact that demethylation of the promoter CpG islands was incomplete in samples treated with 0.2 μmol/l 5-aza-CdR and 0.33 μmol/l TSA, the expression of this gene was either induced (Fig. 2B). The minimum concentration of 5-aza-CdR (with TSA 0.33 μmol/l) which could induce mRNA expression was 0.2 μmol/l in this study. But RT-PCR could not detect LRP15 mRNA expression in HeLa cells treated with TSA alone (data not shown).

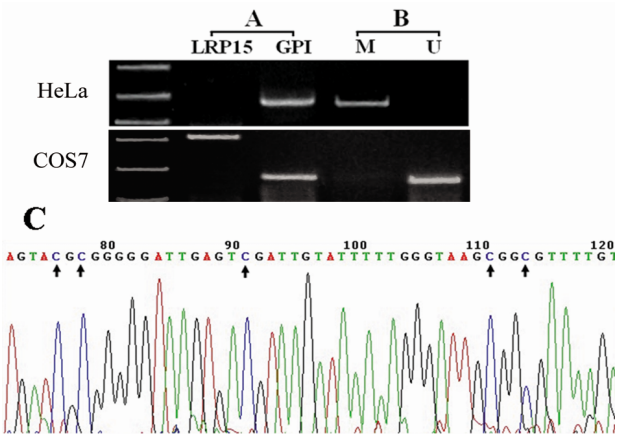

Fig. 1. Methylation analysis and expression of LRP15 in HeLa and COS7 cells. (A) LRP15 mRNA expressionin HeLa and COS7 cells. (B) Methylation status of the LRP15 promoter in HeLa and COS7 cells. (C) Part of the results of DNA sequencing of the MS-PCR products. Completely methylated cytosines (black arrow) in HeLa cells were not converted to thymidine following bisulfite treatment.

Overexpression of LRP15 inhibits HeLa cell proliferation

In response to overexpression of LRP15, we observed profound alterations in cell proliferation. As shown in Fig. 3A, cell proliferation was markedly inhibited after LRP15 transfection. The effects of overexpression of LRP15 on cell differentiation were analyzed by flow cytometry. Overexpression of LRP15 induced an increase of about 10% in cell numbers in G_2/M phase, and a decrease of about 10% in cell numbers in S phase.

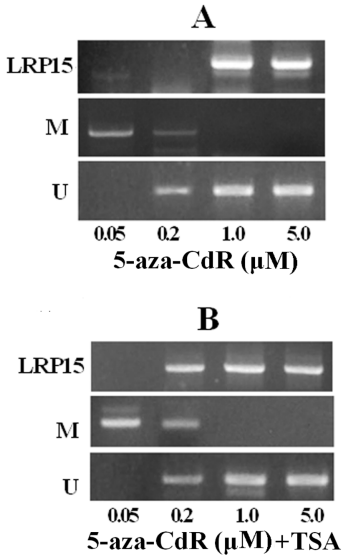

Fig. 2. Expression and methylation status of LRP15 gene in drug treated HeLa cells. (A) Expression and methylation status of LRP15 gene in HeLa cells treated with various concentration of 5-aza-CdR alone. (B) Expression and methylation status of LRP15 gene in HeLa cells treated with various concentration of 5-aza-CdR and TSA (0.33 μmol/L).

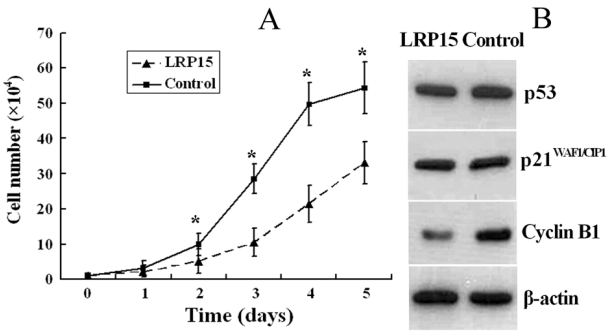

Fig. 3. Effect of LRP15 overexpression on HeLa cells. (A) HeLa-LRP15 cell proliferation was markedly inhibited compared with HeLa-pcDNA (control). *P < 0.05. (B) The protein levels of p53 and $p21^{WAF1/CIP1}$ were not different in the HeLa-LRP15 cells compared with control cells, while cyclin B1 was much lower in HeLa-LRP15 cells compared with control cells.

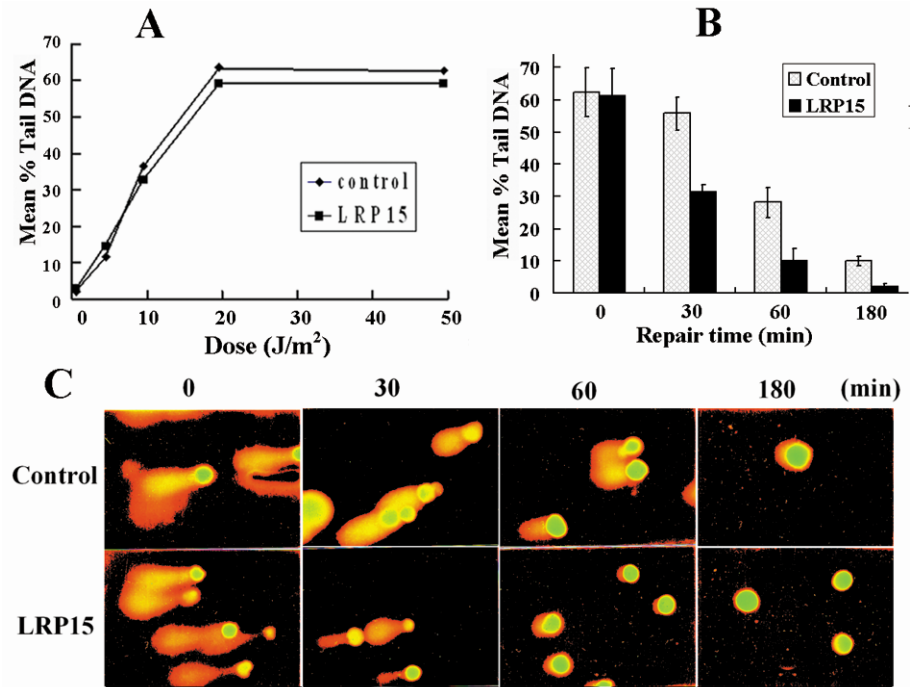

Fig. 4. Comparison of data from Comet assay. (A) Dose response of HeLa cells to UV-radiation. (B) The Mean % Tail DNA of comet assay after exposure to 20 J/m^2 UV. Mean ± S.E.M. of triplicates scores are shown. (C) Representative images of cells following Comet assay. (all magnifications 400 ×).

Overexpression of LRP15 decreases cyclin B1

The expression levels of p53, p21$^{WAFI/CIPI}$ and cyclin B1 were measured by Western blot analysis. As shown in Fig. 3B, the expression levels of p53 and p21$^{WAFI/CIPI}$ were not different in the HeLa-LRP15 cells compared with control cells, while cyclin B1 was much lower in the cells with overexpression of LRP15 compared with control cells.

More efficient DNA repair in LRP15-positive cells after UV exposure

HeLa-LRP15 and HeLa-pcDNA cells were exposed to UV radiation. DNA repair at different time points after irradiation was assessed using the Comet assay. After exposure to UV radiation, HeLa-LRP15 showed an initial dose-dependent increase in Mean % Tail DNA (Fig. 4A). But exposure to either 20 J/m^2 or 50 J/m^2, the Mean % Tail DNA were similar. As early as 30 min and continuing through 3 h after exposure to 20 J/m^2 UV, as shown in Fig. 4B, scoring of 100 cells/slide indicated that the LRP15-positive cells (HeLa-LRP15) showed shorter comet tails as compared with the LRP15-negative cells (HeLa-pcDNA; $P < 0.05$), consistent with greater DNA repair. By 3 h, the vast majority of HeLa-LRP15 cells were almost complete repaired. In contrast, < 10% of HeLa-pcDNA cells were almost complete repaired. The photographs shown in the figures below are representative views of the slides for HeLa-LRP15 and HeLa-pcDNA cells (Fig. 4C). The results suggest that DNA repair is more rapidly completed and hence more efficient in LRP15-positive cells.

DISCUSSION

We cloned LRP15 from lymphocytes for the purpose of finding leukemia relapse related gene, but there was no difference in LRP15 mRNA expression between newly diagnosed and relapsed patients with acute myeloid leukemia (3, 4). Bioinformatic data showed that LRP15 might be regulated by DNA methylation and important for DNA repair (2).

Cancer cells keep on accumulating genetic changes that allow them to evading various chemotherapeutic drugs and hence becoming increasingly dangerous (8, 9). We tried to answer some of the questions by exploring the role of methylation mediated gene silencing in the novel gene LRP15 in the present study. We arised a question whether hypermethylation of LRP15 resulted in the loss of gene expression. The present study shows that methylation is one of the important determinants, because expression of LRP15 gene correlates well with hypermethylation of the promoter sequences. In other words, it demonstrates that promoter aberrant methylation of LRP15 is an important mechanism for inactivation of this gene in HeLa cells.

In order to test whether other epigenetic mechanisms such as partial methylation and histone deacetylation can play a role, we examined the expression of LRP15 gene after treatment with 5-aza-CdR and TSA. DNA hypermethylation-mediated gene silencing is closely associated with histone modifications such as methyl-H3-K9. Although DNA hypermethylation is essential to maintain repressive state of histone code, histone modifications precede DNA hypermethylation in si-

lencing specific genes (10-12). In the present study, although demethylation of the promoter CpG islands was incomplete in HeLa cells treated with 0.2 μmol/l 5-aza-CdR and 0.33 μmol/l TSA, the expression of this gene was either activated. But it could not be induced in the cells treated with 0.2 μmol/l 5-aza-CdR alone. This result suggests that key histone modifications, either by direct or indirect involvement of promoter methylation, also play a role in down-regulating LRP15 gene expression in this cancer type. It is therefore assumed that LRP15 silencing may be associated with the state of genomic instability in HeLa cells.

Bioinformatic data indicate that LRP15 is involved in regulating genes that encode components of the DNA damage checkpoint and repair pathways, which imply that this gene may contribute to cell cycle regulation. This hypothesis was demonstrated in the present study. We successfully established of a cell line, HeLa-LRP15, overexpression of LRP15, which allowed us to analyze cellular events occurring in LRP15-positive cells. The present study revealed that overexpression of LRP15 inhibited HeLa cell proliferation and induced HeLa cell arrest in G_2/M phase.

As we know now that the cell cycle progression is critically regulated by sequential activation of cyclin-dependent kinases (CDKs). Cyclins B1 is involved in regulation of the G_2/M phase of the cell cycle. Cyclin B1 is synthesized through the S and G_2 phases and associates with cdc2 to become an inactive complex, called the pre-mitosis-promoting factor (pre-MPF). So loss of cyclin B1 may result in G_2/M phase cell cycle arrest. The result of Western blot showed, although the expression levels of p53 and $p21^{WAFI/CIPI}$ were not different, the expression levels of cyclin B1 were decreased by overexpression of LRP15 compared with control cells. It indicates that decreased levels of cyclin B1 may mediate LRP15-induced cell cycle arrest at the G_2-to-M checkpoint.

The accurate regulation of the cell cycle is important for DNA repair. Cells treated with DNA damaging agents, such as γ radiation, ultraviolet (UV) radiation, adriamycin or cisplatin, coordinately arrest their cell cycle progression at the G_1/S phase, the S phase and the G_2/M phase to allow times for repairing the damage. Cellular machineries that mediate cell cycle arrest are called cell cycle checkpoints, which monitor DNA status and ensure the completion of the previous phase in the cell cycle before advancing to the next phase (13-15).

Previous data suggested DNA irradiated with artificial UV radiation would accumulate single-strand breaks, double- strand bleaks, and cyclobutane pyrimidine dimers. The damage would be repaired by DNA repair system. And nucleotide excision repair has been identified as the major DNA repair pathways (16). Single cell gel electrophoresis (SCGE), also known as the Comet assay is a straightforward visual method for the detection of DNA damage in interphase cells. In this study, we used the classic Comet assay to detect repair phase of DNA breaks. After exposure to UV radiation, HeLa-LRP15 showed an initial dose-dependent increase in DNA fragmentation, which may reflect the mechanism involved in the repair of damaged DNA. As early as 30 min and continuing through 3 h after exposure to UV radiation, the LRP15-positive cells showed shorter comet tails as compared with the LRP15-negative cells. By 3 h, the vast majority of the cells with overexpression of LRP15 were almost complete repaired. The results suggest that DNA repair is more rapidly completed and hence more efficient in LRP15-positive cells after UV exposure. And we assume that DNA repair deficiency maybe exists in HeLa cells for the absence of LRP15. Genetic instability as a consequence of DNA repair deficiency is generally regarded as an enabling trait for cancerogenesis (17), suggesting that LRP15 might indeed play an important role in the maintenance of genetic stability and hence the prevention of tumorigenesis.

In conclusion, we demonstrated that the promoter of LRP15 was hypermethylated in HeLa cells, and lost its transcription. After 5-aza-CdR treatment, with or without TSA, the silencing of LRP15 gene by de novo methylation can be reversed. It indicates that the expression of LRP15 is controlled by methylation in its promoter in HeLa cells. Our study also showed, for the first time, that in response to UV irradiation, LRP15 confered resistance to overt UV damage and accelerated the DNA repair rate. These changes are accompanied by decreasing of the protein levels of cyclin B1 and cell cycle arrest in G_2/M phase. Our study indicates that LRP15 is a new candidate for investigating the mechanism of cancer development. Activating the effect of LRP15 may also provide a novel therapy for human cervical cancer.

MATERIALS AND METHODS

Materials

The vectors including pcDNA3.1 and pGEMT-easy, PCR product extraction kit, plasmid purification kit and DNA Prep Kit were purchased from Promega. The transfection reagent Super-Fect was purchased from Qiagen. LA Tag DNA polymerase and various restriction enzymes were purchased from TaKaRa. Fetal calf serum (FCS), Dulbecco's modified Eagle's medium (DMEM), proteinase K, RNA extraction reagent Trizo1 and G418 were purchased from Gibco BRL. Superscript II RNase reverse transcriptase was purchased from Invitrogen. The DNA demethylating agent, 5-aza-2'-deoxycytidine (5-aza-CdR), and the histone deacetylase inhibitor, trichostatin A (TSA), were purchased from Sigma. All antibodies, including anti-human cyclin B1, p53, $p21^{WAF1/CIP1}$ and β-actin used for the Western blot analysis and the chemiluminescence luminol reagent were purchased from Santa Cruz Biotechnology.

Cell culture

Human cervical cancer cell line (HeLa) and monkey kidney cell line (COS7) were purchased from ATCC. The cells were maintained in DMEM supplemented with 10% FCS and 100 U/ml penicillin-streptomycin in a humid atmosphere with 5% CO_2 at 37°C.

Reverse transcription PCR (RT-PCR)

The expression of LRP15 mRNA was examined by RT-PCR, which was performed as we described before (3, 4). The gene specific primers designed with DNAStar v5. 0 were used for the PCR step: sense, 5'-TGA GCC ACG AGG ATG GAG CA-3', and antisense, 5'-CAT TTT TGG ACA GGT TGA GAA-3'. The LRP15 gene transcripts were amplified in a GeneAmp 9600 thermal cycler (Applied Biosystems) in 30 cycles, each consisting of 30 s at 94°C for denaturation, 30 s at 59°C for annealing and 30 s at 72°C for extension, follow by an extension for 5 min at 72°C. To ensure that equal amounts of reverse transcribed cDNA were applied to the PCR reaction, the GPI gene was also included in the PCR as a control as we described before (3, 4).

DNA preparation and methylation-specific PCR (MS-PCR)

Genomic DNA was extracted by the DNA Prep Kit. DNA methylation patterns in CpG islands of LRP15 gene were determined by chemical modification with sodium bisulphite as described previously (18). Following primer pairs for LRP15 gene, methylated CpG site, sense: 5'-GAG TAG GGT TCG TAG CGG TCG TC-3', and antisense: 5'-TTA ACT CCC GAC GAA AAC GAC G-3'; unmethylated CpG site, sense: 5'-GGA GTA GGG TTT GTA GTG GTT GTT-3', and antisense: 5'-AAC TCC CAA CAA AAA CAA CAA CA-3'. The PCR reactions were cycled under the following conditions: preheat at 94°C for 3 min, followed by 35 cycles (94°C for 40 s, 60°C for 40 s in methylated gene or 59°C for 40 s in unmethylated gene, 72°C for 45 s) and a final extension at 72°C for 5 min. To verify the PCR results, representative bands from each target were gel-purified and cloned into pGEMT-vector, followed by DNA sequencing provided by ShengGong Co.

Demethylation assay

HeLa cells were plated in complete medium and treated with various concentrations of the DNA demethylating agent, 5-aza-CdR (0.05, 0.2, 1.0 and 5.0 μmol/l) for 72 h, or treated with various concentrations of the histone deacetylase inhibitor, TSA (0.1, 0.33, 1.0 and 3.3 μmol/l) for 72 h, or treated with 5-aza-CdR (0.05, 0.2, 1.0 and 5.0 μmol/l) and TSA (0.33 μmol/l) for 72 h. The medium and the drug were replaced every 24 h. At the end of the treatment period, the medium was removed and RNA was extracted for RT-PCR and DNA for MS-PCR.

Construction of LRP15 expression vector and stable transfection

The entire coding region of LRP15 was generated from cDNA extracted from the mononuclear cells of normal bone marrow by RT-PCR. The PCR products were subcloned into pGEMT-easy vector and sequenced to ensure that no misincorporated mutations were introduced into PCR products during amplification. The full coding region of LRP15, which was a 794 bp fragment, was digested with KpnI and BamHI from pGEM-LRP15 and was inserted into the same sites of the pcDNA3.1 vector. The construction was confirmed by enzymatic digestion, and was sequenced to verify the correct reading frame. Both the construct (pcDNA-LRP15) and pcDNA3.1 were transfected into HeLa cells by the SuperFect transfection reagent. The HeLa cells transfected with pcDNA-LRP15 was named HeLa-LRP15; and with pcDNA3.1 was named HeLa-pcDNA which was used as a negative control. Two days after transfection, the cells were treated with G418 at 1 mg/ml for 3 weeks and then were continuously cultured with 0.5 mg/ml G418. For a cell proliferation assay, the cells transfected with LRP15 or empty vector were plated onto 24-well plates at 1×10^4 cells/well in the culture medium and the medium was changed every 2 days. After washing with PBS two times, the cells were trypsinized and then counted by the Trypan Blue exclusion method using a hemocytometer every 24 h for 5 days.

Cell cycle analysis

To analyze an effect of LRP15 on cell cycle progression, we incubated HeLa-LRP15 and HeLa-pcDNA cells with serum-free DMEM for 72 h and then were continuously cultured with 10 % FCS for 24 h. DNA content profile of a given population was determined by flow cytometry (12). Treated cells were fixed with 100 % ethanol overnight and treated with 0.25 μg/ml RNase. Nuclei were stained with 50 μg/ml propidium iodide. Cells were analyzed with a flow cytometer (Becton Dickinson).

Western blot analysis

The expression of the cell cycle-related proteins including p53, $p21^{WAF1/CIP1}$, cyclin B1 and β-actin were examined by Western blot analysis, as we described previously (19, 20), using specific antibodies.

UV-irradiation of HeLa cells

Both HeLa-LRP15 and HeLa-pcDNA cells were washed and resuspended in 2 ml of saline buffer. Two ml of the cells culture at an approximate cell density of 2×10^6 cells/ml was exposed to UV light in a 4.5 cm diameter petridish. Source of UV radiation was a 15 W phillips TUV germicidal lamp (wavelength 254 nm) with a fluence of 0.06 J/m^2/s. Aliquots of the cell culture were removed after irradiated at 0, 5, 10, 20 and 50 J/m^2. Samples were obtained at the indicated recovery times after irradiation. Alternatively Comet assay was used to examine samples immediately for strand breaks.

Single cell gel electrophoresis (Comet assay)

A standard protocol for Comet preparation and analysis was adopted (21). For evaluation of DNA damage, 100 cells per subject were analyzed at 400 × magnification under a fluorescent microscope equipped with a 540 nm excitation filter and a 590 nm barrier filter. The Mean % Tail DNA was meas-

ured by using the CometScore software from three independent experiments and their average was presented as mean ± S.E.M.

Statistical analysis

Statistical analysis was performed using Stata7.0 software. For the cell proliferation assay and Comet assay, experiments were performed in triplicate, and the results were expressed as the mean ± S.E.M. of three independent experiments. Significance of the differences in mean values was determined using the one-way ANOVA, and $P < 0.05$ was considered to be statistically significant.

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