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MALAT1 Decreases the Sensitivity of Head and Neck Squamous Cell Carcinoma Cells to Radiation and Cisplatin

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Abstract. *Background/Aim: Two-thirds of head and neck squamous cell carcinoma (HNSCC) patients present with locally advanced (LA) stages and have a poor survival rate. The aim of this study was to investigate the roles of the long non-coding RNAs MALAT1 on radiation and cisplatin sensitivity of HNSCC cells. Materials and Methods: Clonogenic, cell viability, and apoptosis assays were performed in cells following MALAT1 knockdown using CRISPR/Cas9 system. Results: MALAT1 was overexpressed in HNSCC cell lines as compared to a non-tumorigenic cell line. The number of colonies formed after radiation was significantly reduced in MALAT1 knockdown cells. The IC_{50} value of cisplatin in MALAT1 knockdown cells was lower than that of the control cells. MALAT1 knockdown resulted in cell cycle arrest at G_2/M phase, DNA damage and apoptotic cell death. Conclusion: MALAT1 knockdown enhanced the sensitivity of HNSCC cells to radiation and cisplatin partly through the induction of G_2/M cell cycle arrest resulting in DNA damage and apoptosis.*

Head and neck squamous cell carcinoma (HNSCC) is one of the most common malignant tumors arising from epithelial cells of the oral cavity, nasal cavity, pharynx, larynx, and hypopharynx. HNSCC is the sixth most common cancer worldwide in which two-third of patients are diagnosed with locally advanced stage. Annually, approximately 700,000 new HNSCC cases and 350,000 death are reported (1). Concurrent chemoradiotherapy (CRT) using platinum-based chemotherapy (*e.g.* cisplatin or carboplatin) and/or surgery are considered a standard treatment for locally advanced (LA) HNSCC patients. Cisplatin is considered as the mainstay chemotherapy agent for CRT in patients with LA-HNSCC. Nevertheless, the majority of LA-HNSCC patients suffer from locoregional recurrence due to CRT resistance, and the overall survival (OS) rate remains poor (2, 3). Therefore, understanding the mechanism of radiotherapy and cisplatin sensitivity in HNSCC is essential to improve survival outcome of LA-HNSCC patients.

Long non-coding RNAs (lncRNAs) are a novel class of non-protein coding transcripts containing >200 nucleotides (4). Increasing evidence has reported the critical roles of lncRNAs in the regulation of multiple cellular processes (5). The molecular mechanism relies on the interaction of lncRNAs with other biomolecules including chromatin, protein and RNA (6). They also play a role in epigenetic regulation through the recruitment of chromatin-modifying complexes to specific genomic DNA targets (7). Moreover, lncRNA can also function as endogenous sponge for other RNAs, such as mRNAs and miRNAs to regulate their stability and translation (8). Alteration of lncRNA functions has been shown to associate with treatment sensitivity of cancer cells through multiple cellular processes, such as chromatin modification, DNA damage and repair mechanism, cell cycle

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Key Words: HNSCC, lncRNA, *MALAT1*, radiation sensitivity, cisplatin sensitivity.

Table I. *Primer sequences for genotyping.*

Name	Primer sequences
MALAT1 (1st PCR)	FW: 5'-GGGAGCAAGTCGCAGGA-3'
	RV: 5'-AAAAGCATTGCCCTTCTATTGG-3'
MALAT1 (2nd PCR)	FW: 5'-GGGAGCAAGTCGCAGGA-3'
	RV: 5'-CACTTCTTGTGTTCTCTTGAGGG-3'

Table II. *Primer sequences for quantitative real time PCR.*

Gene	Primer sequences
HOTAIR	FW: 5'-GGTAGAAAAAGCAACCACGAAGC-3'
	RV: 5'-ACATAAACCTCTGTCTGTGAGTGCC-3'
MALAT1	FW: 5'-ATGCGAGTTGTTCTCCGTCT-3'
	RV: 5'-TATCTGCGGTTTCCTCAAGC-3'
TUG1	FW: 5'-TAGCAGTTCCCCAATCCTTG-3'
	RV: 5'-CACAAATTCCCATCATTCCC-3'
MEG3	FW: 5'-GCATTAAGCCCTGACCTTTG-3'
	RV: 5'-TCCAGTTTGCTAGCAGGTGA-3'
PVT1	FW: 5'-CCGACTCTTCCTGGTGAAGC-3'
	RV: 5'-GTATGGTCAGCTCAAGCCCA-3'
GAPDH	FW: 5'-ACCACAGTCCATGCCATCAC-3'
	RV: 5'-TCCACCACCCTGTTGCTGTA-3'

control, and mRNA processing and stability (5, 9). Recently, the differential expression of lncRNA between HNSCC and their adjacent normal tissues has been revealed. The study reported that down-regulation of two lncRNAs, *lnc-LCE5A-1* and *lnc-KCTD6-3*, was significantly associated with poor prognosis of HNSCC patients (10).

In addition to the aforementioned lncRNAs, *MALAT1* (metastasis-associated lung adenocarcinoma transcript 1) also plays critical roles in cancer carcinogenesis and progression. *MALAT1* has been shown to be dramatically up-regulated in various types of cancer including HNSCC (11). Up-regulation of *MALAT1* was associated with poor prognosis and promoted cell proliferation and migration of TSCC (tongue squamous cell carcinoma) and LSCC (laryngeal squamous cell carcinoma) (12). Down-regulation of *MALAT1* in HR-HPV$^+$ cervical cancer increased its sensitivity to radiation via sponging of miR-145 (13). However, a role of *MALAT1* in regulating radiation and cisplatin sensitivity of HNSCC cells, which is a key treatment of LA-HNSCC patients, has not been explored.

Materials and Methods

Cell culture. FaDu (hypopharyngeal carcinoma) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Ca9-22 (oral squamous cell carcinoma) cells were obtained from the Japanese Collection of Research Bioresources (JCRB, Osaka, Japan). FaDu and Ca9-22 cells were cultured in Eagle's Minimum essential medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, ST. Louis, MO, USA). HaCaT (non-tumorigenic human skin keratinocyte) cells (14) were cultured in Dulbecco's Modified Eagle's Medium (Invitrogen) supplemented with 10% fetal bovine serum. All cells were maintained in a humidified 95% air incubator with 5% CO_2 at 37˚C. Cells were regularly tested for mycoplasma contamination before use.

Stable MALAT1 knockdown. FaDu cells were cotransfected with pDECKO_*MALAT1*_C and lentiCas9-Blast plasmids (Addgene, Watertown, MA, USA) by Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. Stable transfected cells were selected by culturing in medium containing blasticidin (10 μg/ml) and puromycin (2 μg/ml) for 7 days. Individual cells were isolated, plated into 96-well plates and cultured for approximately 15 to 20 days. For genotyping, the genomic DNA was extracted using QIAamp DNA micro kit (QIAGEN, Hilden, Germany). Two primer sets were used for genotyping by PCR (Table I). The 1st PCR primers were designed to flank the genomic target region of CRISPR/Cas9. The absence of wild type allele was confirmed by the 2nd PCR primers located in the deleted region.

RNA extraction and real-time PCR. Total RNA was extracted using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The quality and quantity of RNA was determined using a NanoDrop 2000C (Thermo Scientific, Wilmington, DE, USA). Complementary DNAs (cDNAs) were synthesized by iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). The expression of *HOTAIR*, *MALAT1*, *TUG1*, *MEG3*, and *PVT1* were examined by real-time PCR with specific primers (Table II) using SYBR Green (Bio-Rad) with the ABI PRISM7500 Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA). Samples were analyzed in triplicate and normalized with GAPDH as a house keeping gene control.

Cell viability. Cells were plated at 1.5×10^4 cells/well in 96-well plates and treated with 0.1, 0.5, 1, 5, 10, and 20 μM of cisplatin for 48 h. Cell viability was assessed by the MTT assay. Briefly, cells were incubated with 0.5 mg/ml of MTT for 4 h at 37˚C in humidified 5% CO_2 incubator. Dimethylsulfoxide (DMSO) was added to dissolve the dark blue formazan. Then the absorbance was measured at 540 nm using a Multiskan GO Microplate Spectrophotometer (Thermo Scientific). The results were calculated as % of cell viability normalized to control.

Clonogenic assay. MALAT1 knockdown and control FaDu cells were cultured in T25 flasks and exposed to 0-, 2-, 4-, 6-, and 8-Gy of 6 MV X-ray using a linear accelerator Clinac iX (Varian Medical System, Palo Alto, CA, USA). The dose homogeneity within the T25 cell culture flasks was ±3% acquired using EBT3 gafchromic film (International Specialty Product, Wayne, NJ, USA). After radiation, cells were immediately plated in 6-well plates at 5×10^2 cells/well and exposed to 0-, 2-, and 4-Gy radiation while 2×10^3 cells/well were exposed to 6- and 8-Gy radiation. Cells were maintained in complete growth medium for 14 days. The formed colonies were fixed with 100% methanol for 20 min and stained with 0.5% crystal violet at room temperature for 1 h. After air dry,

the stained colonies were photographed and counted using Image J. The surviving fraction was defined as the number of colonies divided by the number of plated cells.

Cell cycle analysis. *MALAT1* knockdown and control FaDu cells were seeded in 60 mm culture dishes at 6×10^5 cells/dish and cultured for 48 h. Cells were then fixed with cold 70% ethanol overnight at 20ºC. After washing twice with PBS containing 2% FBS, cells were stained with Propidium Iodide (PI)/RNase solution for 15 min in the dark. The stained cells were analyzed using a BD FACSCalibur™ flow cytometer (BD Bioscience, San Jose, CA, USA). The percentage of DNA content in each of cell cycle phase was quantified using BD FACSDiva software version 6.1.1 (BD Bioscience).

Apoptosis assay. *MALAT1* knockdown and control FaDu cells were seeded in 60 mm culture dishes at 6×10^5 cells/dish and cultured for 24 h. Cells were either exposed to X-ray (8-Gy) or cisplatin (8 μM) for 48 h before harvesting by trypsinization. After washing twice with PBS, cell pellets were collected by centrifugation and incubated with FITC-labeled annexin V and Propidium Iodide (PI) for 15 min. The stained cells were analyzed using a BD FACSCalibur™ flow cytometer and quantified by BD FACSDiva software version 6.1.1 (BD Bioscience).

Western blotting. Cells were harvested and lysed with a modified radioimmunoprecipitation assay lysis buffer (50 mM Tris-HCl pH7.4, 150 mM NaCl, 1mM EDTA, 1% Triton X-100, 1 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF and protease inhibitor cocktail). After 20 min of incubation on ice, cells were centrifuged at 16,000×*g* for 20 min at 4˚C. The supernatants were collected, and protein concentration was measured by the BCA (Bicinchoninic acid) method. Equal amount of proteins was mixed with Laemmli buffer and heated at 95˚C for 5 min. Proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and subsequently transferred to a nitrocellulose membrane by electro-blotting. Non-specific binding was blocked by incubating the membrane with 5% non-fat dry milk in tris-buffered saline, 0.1% tween (TBST). The membranes were incubated with the indicated primary antibodies overnight at 4˚C and then washed 5 times with TBST before the incubation with HRP-conjugated secondary antibody at room temperature for 1 h. After washing with TBST, the signals were detected using Luminata Crescendo Western HRP Substrate exposed on Amersham hyperfilm ECL (GE Healthcare Life Sciences, Pittsburgh, PA, USA). The densitometry analysis was performed using ImageJ software.

The radiobiological response parameters. Dose-response curves for cell viability were fit using a Lineal-quadratic model for the surviving fraction (SF) of cells after an acute dose D is given by SF (D) = exp (-αD-βD^2). α and β were fit using the least square method from the data on response to intermediate dose considering realistic values for α/β. Nonlinear regression analysis was performed on survival curves using GraphPad Prism version 7.0.

Statistical analysis. All data were expressed as means±standard error of means (means±SEM). The statistical analysis was analyzed by using the statistical software package, GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA). Data in each group were compared using a two-tailed unpaired student's *t*-test. *p*-Values <0.05 were considered as statistically significant.

Results

Dysregulation of lncRNAs expression in head and neck squamous cell carcinoma cells. We first examined the expression of five lncRNAs including *MALAT1*, *HOTAIR*, *TUG1*, *MEG3* and *PVT1* in two HNSCC cell lines (Ca9-22 and FaDu) as compared to the non-tumorigenic human skin keratinocyte cell line (HaCaT) by quantitative RT-PCR. As shown in Figure 1A, the expression of *MALAT1* was significantly higher in Ca9-22 ($p<0.05$) and FaDu ($p<0.0001$) cells as compared to HaCaT cells. Significantly higher expression levels of *PVT1* were observed only in FaDu cells ($p<0.05$), but not in Ca9-22 cells. In contrast, the expression of *HOTAIR* and *MEG3* was significantly lower in both HNSCC cell lines ($p<0.01$ and $p<0.0001$). The results also showed significantly lower expression levels of HOTAIR in FaDu cells as compared to Ca9-22 cells ($p<0.01$). The expression of *TUG1* was significantly lower only in FaDu cells ($p<0.05$). Next, the expression levels of these five lncRNAs in Ca9-22 and FaDu cells were compared (Figure 1B). *MALAT1* had the highest expression in both Ca9-22 and FaDu cell lines. The expression levels of *MALAT1* were approximately 30- and 60-fold higher than *MEG3* and *HOTAIR* in Ca9-22 and FaDu cells, respectively. Therefore, *MALAT1* was chosen to further investigate its role on the sensitivity to radiation and cisplatin of FaDu cells.

MALAT1 knockdown increased the sensitivity of FaDu cells to radiation and cisplatin. To investigate the roles of *MALAT1* in FaDu cells, we established stable *MALAT1* knockdown FaDu cells by using dual excision CRISPR knock out system (DECKO) as described in Materials and Methods (15). The DECKO system adapts the CRISPR/Cas9 approach to silence *MALAT1* gene by deleting the major promoter area of *MALAT1* (Figure 2A). The stable transfected clones were selected under puromycin and blasticidin (Figure 2B). To determine the excision of *MALAT1* promoter in an individual clone, genomic PCR was performed using primers flanking the genomic target region of CRISPR/Cas9 (1st PCR). As shown in Figure 2B, the PCR products from non-transfected and control cells produced only an 871 bp band indicating the presence of intact *MALAT1* promoter. In contrast, a 201 bp PCR products was detected only in *MALAT1* knockdown clone A and B suggesting the deletion *MALAT1* promoter in these clones. We further confirmed the knockdown by performing an additional genomic PCR using primers located in the deleted region (2nd primer). The 188 bp PCR product representing WT allele was detected in all samples. This result indicated that *MALAT1* knockdown clones A and B were heterozygous knockout FaDu cells. Next, the expression of lncRNA *MALAT1* in clone A and B was examined. The expression level of *MALAT1* was significant reduced by approximately 50% and 80% in clone A and clone B as

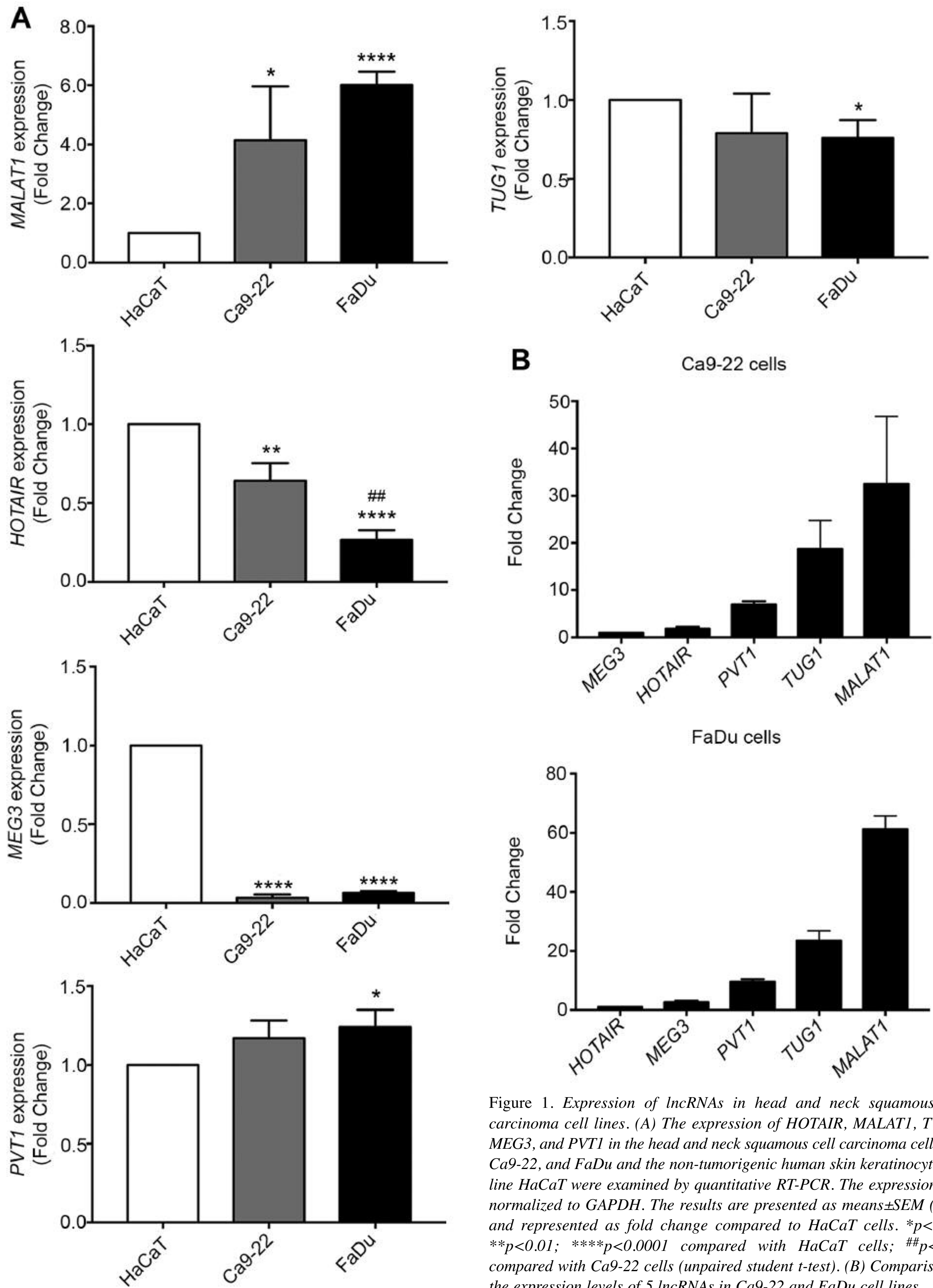

Figure 1. *Expression of lncRNAs in head and neck squamous cell carcinoma cell lines. (A) The expression of HOTAIR, MALAT1, TUG1, MEG3, and PVT1 in the head and neck squamous cell carcinoma cell lines Ca9-22, and FaDu and the non-tumorigenic human skin keratinocyte cell line HaCaT were examined by quantitative RT-PCR. The expression was normalized to GAPDH. The results are presented as means±SEM (n=3) and represented as fold change compared to HaCaT cells. *p<0.05; **p<0.01; ****p<0.0001 compared with HaCaT cells; ##p<0.01 compared with Ca9-22 cells (unpaired student t-test). (B) Comparison of the expression levels of 5 lncRNAs in Ca9-22 and FaDu cell lines.*

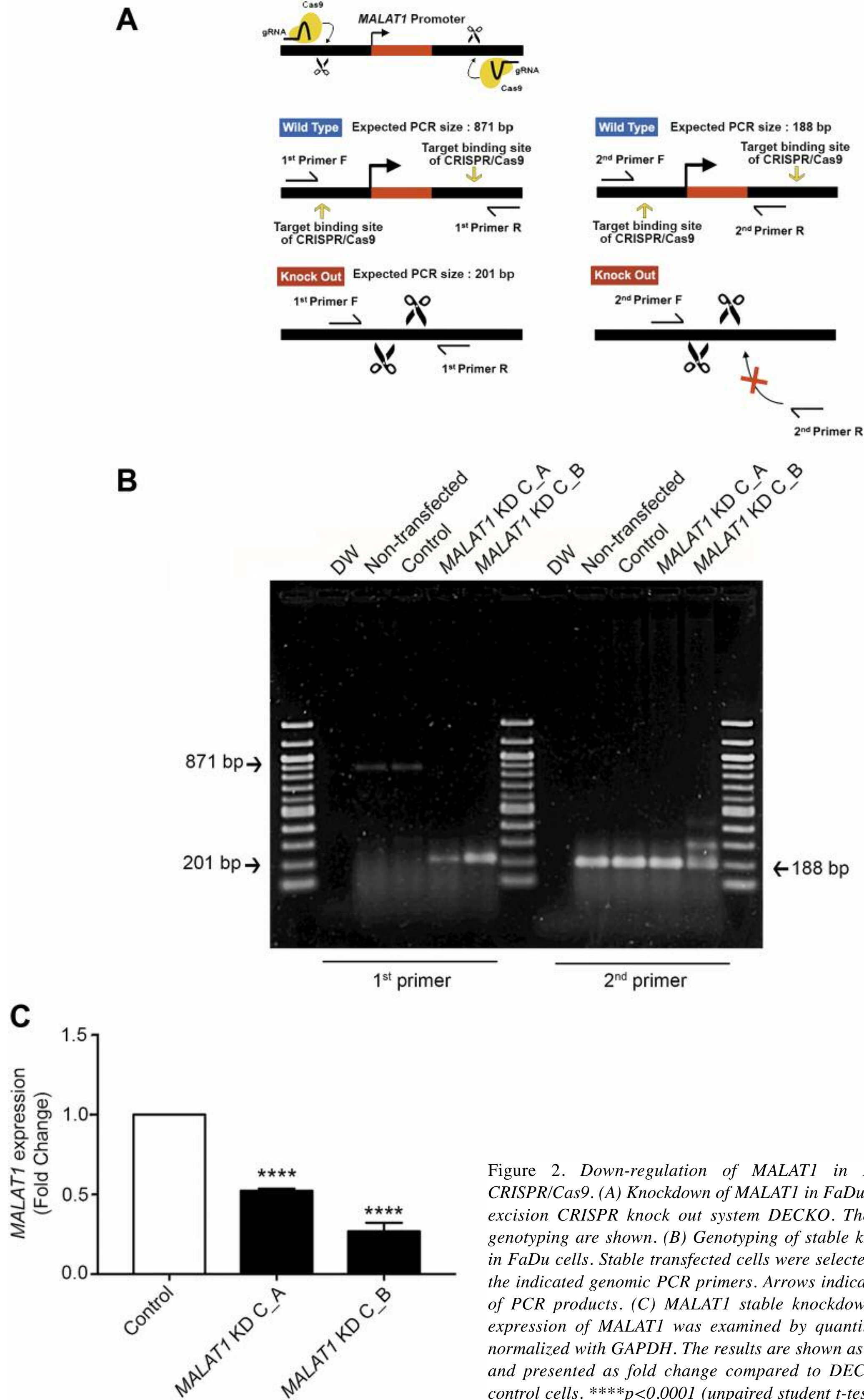

Figure 2. *Down-regulation of MALAT1 in FaDu cells using CRISPR/Cas9. (A) Knockdown of MALAT1 in FaDu cells using the dual excision CRISPR knock out system DECKO. The PCR primers for genotyping are shown. (B) Genotyping of stable knockdown MALAT1 in FaDu cells. Stable transfected cells were selected and genotyped by the indicated genomic PCR primers. Arrows indicate the expected size of PCR products. (C) MALAT1 stable knockdown FaDu cells. The expression of MALAT1 was examined by quantitative RT-PCR and normalized with GAPDH. The results are shown as mean ± SEM (n=3) and presented as fold change compared to DECKO targeting GFP control cells. ****p<0.0001 (unpaired student t-test).*

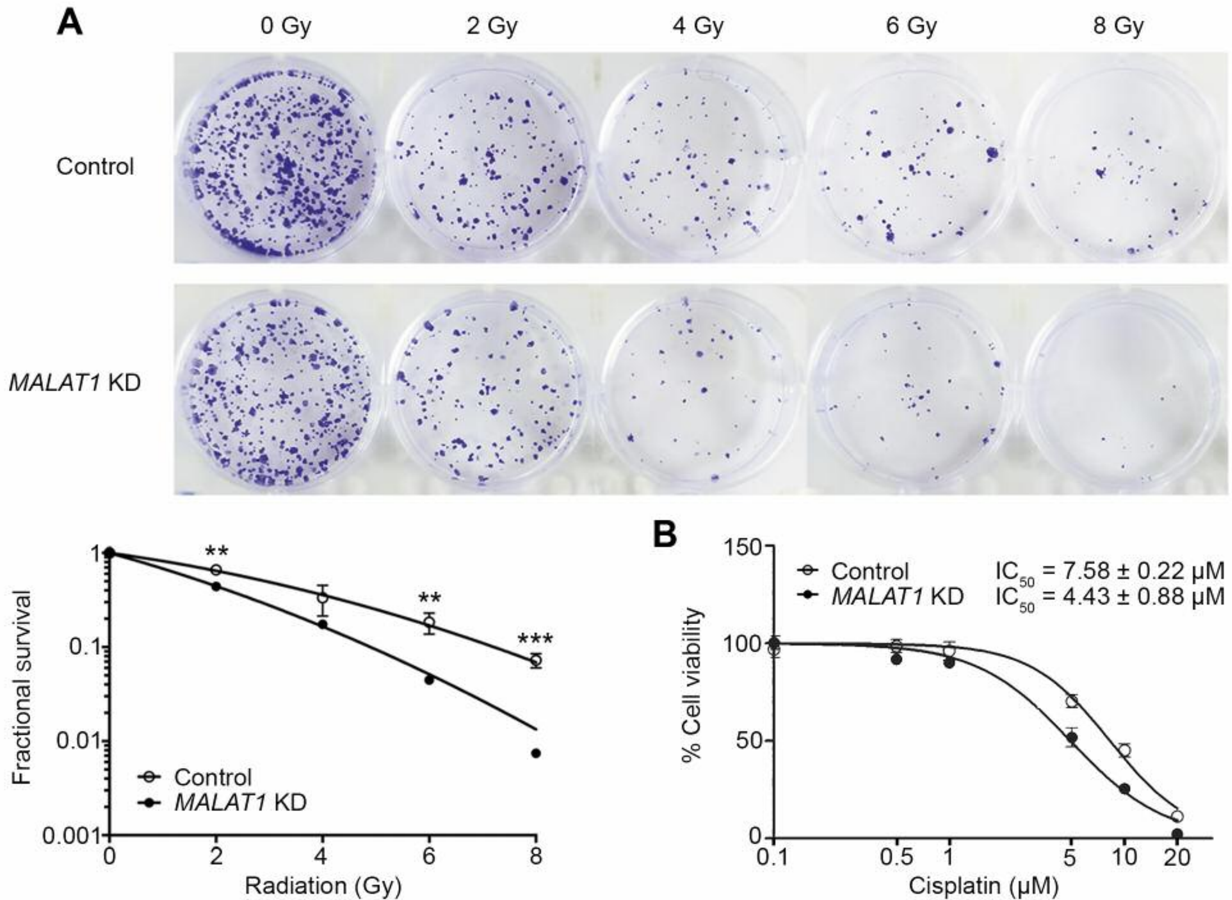

Figure 3. *Down-regulation of MALAT1 increases sensitivity of FaDu cells to radiation and cisplatin treatments. (A) Knockdown of MALAT1 increases sensitivity of FaDu cells to radiation. Knockdown MALAT1 and control cells were exposed to the indicated doses of radiation before reseeded in 6-well plates. After 14 days, the formed colonies were fixed, stained and counted. The fractional survival curves were calculated from the number of colonies in each doses of radiation. (B) MALAT1 knockdown increased the sensitivity of FaDu cells to cisplatin treatment. Knockdown MALAT1 and control cells were incubated with various concentrations of cisplatin for 48 h. Cell viability was assessed by the MTT assay and the IC_{50} values were calculated. The results are presented as means±SEM (n=3). **$p<0.01$; ***$p<0.001$ compared with control cells (unpaired student t-test).*

compared to control cells, respectively ($p<0.0001$) (Figure 2C). Therefore, *MALAT1* knockdown clone B was chosen for further experiments.

To determine the involvement of *MALAT1* in the sensitivity of FaDu cells to radiation and cisplatin, clonogenic survival and cell viability assays were performed. As shown in Figure 3A, after culturing for 14 days, the number of colonies were decreased with increasing doses of radiation. Interestingly, the numbers of the colonies were further decreased in *MALAT1* knockdown cells. The number of colony formation was counted and calculated for fractional survival. The survival of *MALAT1* knockdown FaDu cells was significant reduced as compared to control cells. The effect of *MALAT1* knockdown on FaDu cell survival was initially observed after radiation at a dose as low as 2 Gy. The radiobiological response parameters for both survival fraction curves were α=0.3612 and β=0.0223 (*MALAT1* knockdown cells) and α=0.1796 and β=0.0194 (control cells). Similarly, *MALAT1* knockdown FaDu cell viability after incubation with cisplatin was significantly decreased as compared to control cells ($p<0.01$) (Figure 3B). The IC_{50} values of cisplatin treatment at 48 h in *MALAT1* knockdown and control FaDu cells were 4.43±0.88 μM and 7.58±0.22 μM, respectively. Collectively, these results suggested that *MALAT1* played a role in radiation and cisplatin sensitivity of FaDu cells. Down-regulation of *MALAT1* enhanced sensitivity of FaDu cells to both radiation and cisplatin treatments.

MALAT1 knockdown caused cell cycle arrest and enhanced radiation- and cisplatin-induced DNA damage. It has been shown that the sensitivity of tumor cells to radiation and chemotherapeutic agents depends on the cell cycle. We

therefore further investigated whether the increase in radiation and cisplatin sensitivity of *MALAT1* knockdown FaDu cells is cell cycle dependent. As shown in Figure 4A, knockdown of *MALAT1* significantly increased the ratio of cells in the G_2/M phase but decreased cells in the G_1 phase as compared to control cells. Next, we investigated the effects of *MALAT1* knockdown on DNA damage after radiation and cisplatin treatment of FaDu cells. The expression of DNA damage marker, γ-H2AX (a phosphorylation of histone variant H2AX at Ser139), was detected by western blotting as mentioned in Materials and Methods. We found that radiation treatment induced DNA damage in dose-dependent manner in both *MALAT1* knockdown and control cells as demonstrated by an increased γ-H2AX expression. Interestingly, the effect of radiation exposure on DNA damage was greater in *MALAT1* knockdown cells (Figure 4B). In addition, cisplatin treatment also showed a dose-dependent increase in DNA damage in FaDu cells. Consistently, higher expression of γ-H2AX was also observed after cisplatin treatment in *MALAT1* knockdown FaDu cells as compared with control cells (Figure 4C). Collectively, our results suggested that suppression of *MALAT1* expression increased the sensitivity of FaDu cells to radiation and cisplatin partly through the induction of cell cycle arrest at the G_2/M phase that leads to DNA damage.

MALAT1 knockdown enhanced apoptotic cell death induced by radiation and cisplatin. Next, we investigated the effects of *MALAT1* knockdown on FaDu cell apoptosis after radiation and cisplatin treatments. As shown in Figure 5, *MALAT1* knockdown did not cause apoptotic cell death in FaDu cells. However, apoptotic death of *MALAT1* knockdown cells was increased significantly after treatment with radiation (8 Gy) and cisplatin (8 μM) for 48 h in (Figure 5A and B). Taken together, our results indicated that *MALAT1* knockdown enhanced the sensitivity of FaDu cells to radiation and cisplatin via cell cycle arrest and DNA damage; thereby, inducing apoptotic cell death.

Discussion

Recent studies have revealed that aberrant expression of lncRNAs is associated with malignant phenotypes including chemoradiotherapy resistance of various types of cancer (16). However, a role of lncRNAs in the regulation of radiation and cisplatin sensitivity of HNSCC remains poorly understood. In the current study, we demonstrated that *MALAT1* was overexpressed in HNSCC cell lines (FaDu and Ca9-22) as compared to the non-tumorigenic human skin keratinocyte cell line HaCaT. *MALAT1* knockdown by CRISPR/Cas9 system increased radiation and cisplatin sensitivity of HNSCC cells through cell cycle arrest at G_2/M phase. This subsequently potentiated DNA damage induced by radiation and cisplatin exposure resulting in apoptotic cell death.

Recently, long noncoding RNAs (lncRNAs) have been discovered in the human genome and play critical roles in cellular processes through the regulation of gene expression (9). Their dysregulated expression has been associated with the progression of various types of cancer. Although the expression profiles and biological roles of lncRNAs in cancer have been revealed, there are a few studies on HNSCC (17, 18). In our study, we compared the expression of *HOTAIR*, *MALAT1*, *TUG1*, *MEG3*, and *PVT1* lncRNAs in the HNSCC cell lines FaDu and Ca9-22 with that in the non-tumorigenic human skin keratinocyte cell line HaCaT. These five lncRNAs have been previously reported to be involved in radiation and cisplatin sensitivity of cancer cells (19). We found that expression of *HOTAIR*, *MEG3*, and *TUG1* was significantly lower, while, the expression of *MALAT1* and *PVT1* was significantly higher in the HNSCC cell lines as compared to HaCaT cells. Our results suggested that these lncRNAs could play critical roles in HNSCC carcinogenesis.

Among these lncRNAs, *MALAT1* was found to be the most up-regulated and highly expressed in the HNSCC cell lines, suggesting its crucial role in HNSCC. A previous study has reported that *MALAT1* played crucial roles in the progression of various types of cancer, and its up-regulation was associated with poor prognosis (20). *MALAT1* has indeed been found to be up-regulated in head and neck squamous cell carcinoma, laryngeal squamous cell carcinoma (LSCC), oral squamous cell carcinoma (OSCC) and especially in metastatic tongue squamous cell carcinoma (TSCC) (12, 21). Consistently, we found that CRISPR/Cas9-mediated *MALAT1* knockdown significantly enhanced radiation and cisplatin sensitivity of FaDu cells since these cells demonstrated lower ability of colony formation and lower the IC_{50} values of cisplatin compared to the control cells. Together with the previous studies, our results suggest that *MALAT1* exhibits an oncogenic function in HNSCC cells. The cytotoxic effects of radiation and cisplatin are mediated by the induction of DNA double-strand breaks (22, 23). In addition, the sensitive of human tumor cells to radiation depends on the cell cycle phase. Cells at mitosis and G2 are most sensitive to radiation; while, cells at G1 and S phases are less sensitive (24). Our results demonstrated that knockdown of *MALAT1* in FaDu cells resulted in cell cycle arrest at the G_2/M phase and markedly enhanced radiation-induced and cisplatin-induced DNA damage as demonstrated by an increase in the phosphorylation of H2AX at Ser139. Moreover, increased apoptosis was noted in *MALAT1* knockdown cells. Few cellular mechanisms related to *MALAT1* function have been reported. In esophageal squamous cells carcinoma, down-regulation of *MALAT1* expression by miR-101 and miR-217 reduced cell proliferation through the up-regulation of p21 and p27 expression leading to cell cycle arrest at the G_2/M phase and the inhibition of Myb-related protein B (B-MYB) expression

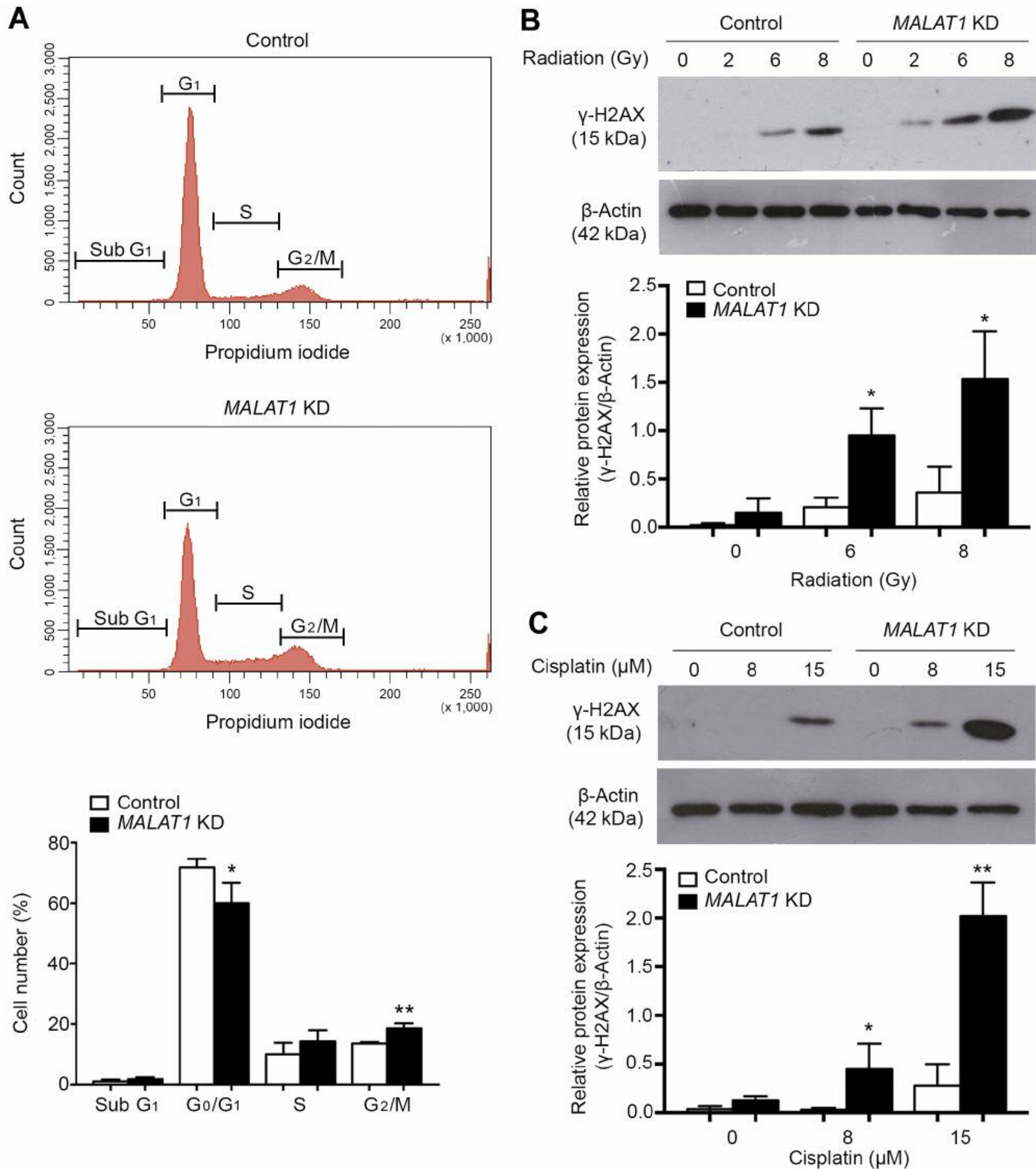

Figure 4. *Down-regulation of MALAT1 causes G_2/M arrest of FaDu cells. MALAT1 knockdown and control FaDu cells were stained with propidium iodide and analyzed by flow cytometry. Bar graph shows the percentages of cell in each cell cycle phase. Data are presented as means±SEM (n=3) *p<0.05, **p<0.01 compared with the control cells (unpaired student t-test). (B) Down-regulation of MALAT1 in FaDu cells increased radiation-mediated DNA damage. MALAT1 knockdown and control FaDu cells were treated with various doses of radiation for 48 h. Total protein lysates were extracted and subjected to western blotting with an anti-γH2AX antibody. β-actin was used as loading control. Bar graph shows relative protein expression that is presented as means±SEM (n=3). *p<0.05 compared with control cells (unpaired student t-test). (C) Down-regulation of MALAT1 in FaDu cells increases DNA damage mediated by cisplatin. MALAT1 knockdown and control FaDu cells were treated with various doses of cisplatin for 48 h. Total protein lysates were extracted and subjected to western blotting with anti-γH2AX. β-actin was used as loading control. Bar graph shows relative protein expression that is presented as means±SEM (n=3). *p<0.05, **p<0.01 compared with control cells (unpaired student t-test).*

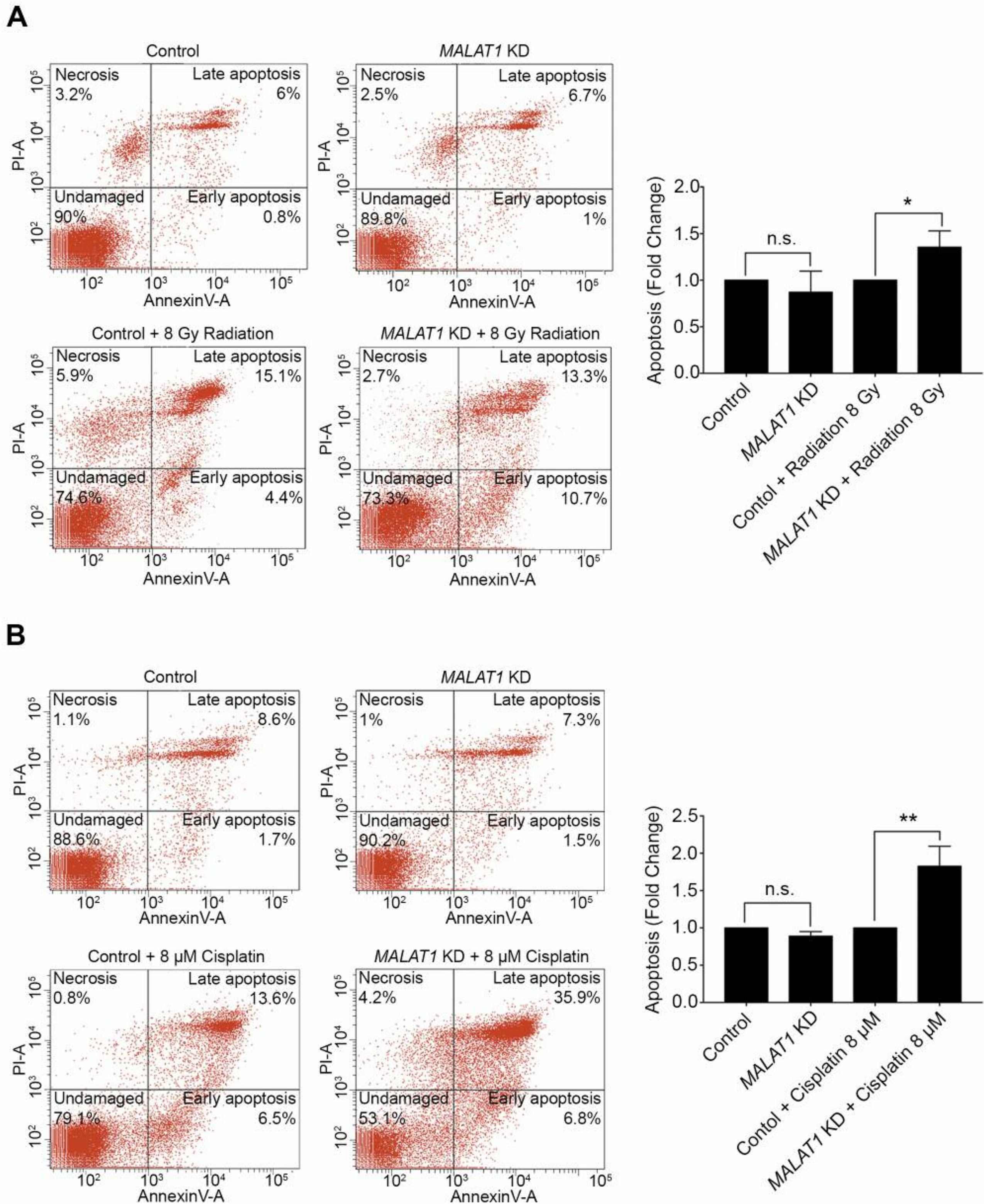

Figure 5. *Down-regulation of MALAT1 induces apoptosis in FaDu cells after radiation and cisplatin treatments. MALAT1 knockdown and control FaDu cells were exposed to indicated doses of radiation (A) and cisplatin (B) for 48 h. Cells were stained with Annexin V-FITC/PI and analyzed by flow cytometry. Bar graphs represent apoptotic cells. Data are mean±SEM (n=3) and expressed as fold change compared to control cells, *p<0.05 compared with the control (unpaired student t-test).*

(25). Moreover, *MALAT1* modulated the sensitivity of cervical cancer to radiation via sponging miR-145 (13), which has been previously reported to increase radiation sensitivity of prostate cancer cells by reducing radiation-induced DNA double strand breaks (26). The expression of miR-145 has been shown to be increased in *MALAT1* down-regulated cervical cancer cells. This enhanced the sensitivity of cervical cancer cells to radiation and subsequently caused cell cycle arrest at the G_2/M phase and apoptosis (13). In addition to radiosensitivity, *MALAT1* also regulates the sensitivity of cancer cells to cisplatin, a well-known chemotherapeutic drug for HNSCC. The expression of *MALAT1* has been found to be increased in cisplatin-resistant NSCLC cells, and its silencing sensitized these cells to cisplatin treatment (27). The roles of *MALAT1* on cell cycle progression at G_2/M were found to be facilitated by the cytoplasmic translocation of hnRNP C protein. Down-regulation of *MALAT1* expression caused cell cycle arrest at the G_2/M phase in HepG2 and HeLa cell lines by compromising the cytoplasmic translocation of hnRNP C protein. This resulted in IRES-dependent translation inhibition and increasing levels of the cell cycle regulatory protein CDK11/PITSLRE^{p58} during mitosis (28). However, the role of *MALAT1* in cell cycle regulation in FaDu cells is still unknown and requires further experiments.

The concurrent chemoradiotherapy (CRT) has been used as a gold standard treatment for locally advanced HNSCC (29). Cisplatin, the platinum-based chemotherapeutic agent, was administered at a dose of 100 mg/m^2 every 3 weeks during radiation therapy (30). Cisplatin has been shown to possess synergistic effect with radiotherapy resulting in an enhancement of radiation sensitivity (31). Although, clinical studies have revealed promising results when cisplatin was combined with radiation (32, 33), the side effects of CRT limited treatment outcomes. Our study showed that down-regulation of *MALAT1* did not cause apoptotic cell death but enhanced radiation and cisplatin sensitivity of HNSCC cells. Therefore, *MALAT1* has the potential to be developed as a new therapy for HNSCC with minimal side effects to normal cells. In order to evaluate the clinical relevance of *MALAT1* on chemoradiotherapy sensitivity, further studies on the effect of *MALAT1* on the sensitivity of cancer cells to the combination of radiation and cisplatin exposure are required. Another important consideration in radiotherapy is the fractionation of treatment. In our experiment, the exposure of HNSCC cells to radiation was performed in a single fraction; in contrast, currently accelerated and fractionated radiotherapy is scheduled. Hyperfractionated radiotherapy has been used in order to reduce the total treatment time, tumor cell regeneration during treatment and late toxicity (29). Therefore, the effects of *MALAT1* in response to relevant clinical fractionation radiotherapy are also needed to be explored in further study.

In summary, higher levels of *MALAT1* were found in FaDu and Ca9-22 cells as compared to HaCaT cells. Down-regulation of *MALAT1* enhanced radiation and cisplatin sensitivity in HNSCC cells by inducing the accumulation of cells at the G_2/M phase leading to DNA damage, and therefore increased apoptotic cell death. Hence, *MALAT1* might serve as a therapeutic agent to enhance radiation and cisplatin sensitivity of HNSCC.

Conflicts of Interest

The Authors declare that they have no competing interests related to this study.

Authors' Contributions

KK, PW, KL and AC conceived the idea and designed the experiments. KC, SK, PC and PT performed experiments and data analysis. KK wrote the draft manuscript. AC and TS contributed to discussion. AC, KL, and NN edited the manuscript. All authors agreed on the final submitted version of the manuscript.

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