Differentially expressed genes in radioresistant nasopharyngeal cancer cells: gp96 and GDF15

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Abstract

Radiotherapy is the major treatment modality for nasopharyngeal cancer (NPC), but in some cases, the disease is radioresistant. We designed this study to identify genes that may be involved in this resistance. We first established two radioresistant subclone cell lines derived from NPC parental cell lines (NPC-076 and NPC-BM1) by treating the cells with four rounds of sublethal ionizing radiation. cDNA microarray analysis was then done, comparing the two resistant cell lines with their corresponding parental cell lines. Seven genes were found to be up-regulated in radioresistant subclones, including gp96 and GDF15, which had shown highest overexpressions. We constructed small interfering RNA plasmids (gp96si and GDF15si) and transfected them into NPC cells to knock down these genes and examine whether this changed their response to radiation. Both gp96si and GDF15si transfectants had radiation-induced growth delay and reduction in colonogenic survival compared with control cells. Knockdown of either gp96 or GDF15 increased the proportion of the cells in G2-M phase, the most radiosensitive phase of the cell cycle. We have therefore identified at least two genes, gp96 and GDF15, involved in radioresistance of NPC cell lines and showed that knockdown of the genes enhances radiosensitivity.

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Introduction

Nasopharyngeal carcinoma is a cancer endemic to southern China and parts of southeast Asia (1–3). Treatment at that point is usually by radiotherapy. The disease tends to be more sensitive to radiation than some other cancers, but success depends mostly on tumor stage. The 5-year survival rate of stages I and II nasopharyngeal cancer (NPC) ranges from 72% to 90% (4, 5). However, in advanced disease, there is a relatively high incidence of locoregional recurrence; 5-year survival rates are ~55% in stage III and 30% in stage IV disease (4, 5). Certain subgroups of NPC are more responsive to treatment. In particular, nonkeratinizing carcinomas (the most common type in endemic areas) have a better prognosis than keratinizing tumors because they respond better to radiotherapy. Even in relatively advanced disease, combined radiochemotherapy may increase survival (4, 5). Nevertheless, radioresistance remains a serious obstacle to successful treatment in many cases.

Our understanding of radioresistance in NPC at a molecular level is limited. Recently, microarray methods have been used to assess genes involved in radioresistance in a number of cell types, including cervical (6), pancreatic (7), oral (8), lung (9), and esophageal cancers (10). However, none of these investigations further confirmed the gene function using molecular cell-based methods. Additionally, there was no overlap in the genes found to be involved in radioresistance in various studies. This may be because of distinct tissue specificity, but it is also possible that there is some fundamental mechanism underlying radioresistance that has not yet been elucidated.

In this study, we established two radioresistant subclones of NPC cell lines to search for genes potentially responsible for resistance by microarray and reverse transcription-PCR (RT-PCR). We further investigated the function of two such genes by looking for enhanced radiosensitivity after gene knockdown with small interfering RNA (siRNA) and by cell cycle analysis.

Materials and Methods

Cell Lines and Culture

Two NPC cell lines, NPC-076 (11) and NPC-BM1 (12), were used. NPC-076 cells were cultured in DMEM (Life Technologies BRL) with 10% FCS and 1% antibiotics. BM1

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Statement of Significance: To search for radioresistant associated genes in nasopharyngeal cancer (NPC), we established two NPC-radioresistant subclone cell lines and identified seven genes that were up-regulated in radioresistant cells. Two of the highest overexpressed genes, gp96 and GDF15, were further studied. Knockdown of gp96 or GDF15 by small interfering RNA significantly caused radiation-induced growth delay, reduced colonogenic survival, and increased the proportion of the cells in G2-M phase, the most radiosensitive phase of the cell cycle. These results suggest the roles of these genes in radioresistance and show that the knockdown of the genes enhances radiosensitivity in NPC.

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cells were cultured in RPMI 1640 (Life Technologies) supplemented with 10% FCS and 1% antibiotics. Cultures were maintained in an incubator at 37°C with humidified 5% CO2.

Establishment of Radioresistant Subclones

The NPC parental cell lines were seeded at a density of 1 × 10^5 per T25 flask (30% to 40% confluency) in complete medium. After 24 h of cell seeding, various doses from 0 to 12 Gy were given to determine the sublethal dose for each cell line. The radiation was delivered at room temperature at 300 cGy/min with a linear accelerator (2100EX, Varian). To establish radioresistant subclone, the NPC cells were received the sublethal dose of irradiation. After treatment, the surviving cells were selected and cultured to produce the first generation of the subline cells. Again, the subline cells received a sublethal dose of irradiation, and the surviving cells were cultured to produce the next generation of subline cells. Up to four generations of NPC cells were produced, and we defined the fourth-generation cells as the radioresistant subclone.

cDNA Microarray Analysis

Total RNA was extracted from the radioresistant cells with TRIzol reagent (Life Technologies) following the manufacturer’s instructions. The concentration, purity, and amount of total RNA were determined by UV spectrometry. Procedures of cDNA microarray analysis were previously described (13–15). Briefly, 20 μg of total RNA was used for labeling and hybridization with the 3DNA 300Array Detection kit (Genisphere), and slides were scanned with a confocal scanner ChipReader (Virtek). Spot and background intensities were acquired with GenePix Pro 4.1 software (Axon Instruments Inc.). To carry out within-slide normalization, the local weighted regress (Lowess) method was used where changes of intensity were assumed to be symmetrical for all spots; thus, normalization was done in each bin of spots. After normalization, we averaged four replicated log ratios of each gene (two from duplicates on each bin of spots. After normalization with the actin level in each individual sample. The level of gene expressions was determined using RT-PCR. Total RNA was extracted from cells with the TRIzol reagent (Life Technologies) following the manufacturer’s instructions. The cDNA was synthesized by incubation of samples in 37°C for 1 h in a final volume of 30 μL containing 50 mmol/L Tris (pH, 8.4), 75 mmol/L KCl, 3 mmol/L MgCl2, 300 ng of RNA, 0.2 μg oligo-dT, 10 mmol/L DTT, 0.5 mmol/L deoxynucleotide triphosphate, 10 units of RNase inhibitor (RNaseOut, Invitrogen Life Technology), and 50 units of reverse transcriptase (M-MLV, Invitrogen Life Technology). To amplify specific genes (primers shown in Table 1), PCR reactions were carried out with 30 cycles of denaturation at 95°C for 40 s, annealing at 56°C to 60°C for 40 s, and extension at 72°C for 1 min. The primer sequences for Gp96 are forward, 5'-AAGGAGAAGACCTGCTGCATG, and reverse, 5'-TG- GGCTCCTCAACAGTCCACTG. The primer sequences for GDF-15 are forward, F: 5'-AGATCAAGACGCGCTG- CACC, and reverse, 5'-CATTCCACAGGCCAGGAGCAA. The PCR products were analyzed using 1.5% agarose gel electrophoresis, stained with ethidium bromide, visualized, and photographed by illuminating with 254 nm UV. The density of each band was determined by Fluorescent Gel Image System (Scion Corporation). The relative density in each sample was expressed as the percentage of the control after normalization with the actin level in each individual sample.

RNA Extraction and RT-PCR Analysis

To determine protein expression, cells were harvested with trypsinization and washed with PBS buffer. Proteins were extracted by incubation for 30 min at 4°C with ice-cold CHAPS lysis buffer [10 mmol/L Tris (pH, 7.4), 1 mmol/L MgCl2, 1 mmol/L EGTA, 150 mmol/L NaCl, 0.5% CHAPS, and 10% glycerol]. Samples were centrifuged at 14,000 rpm for 30 min, and the supernatant was harvested for protein quantification and Western blot analysis. For the latter, 20 μg of protein was prepared. All samples were boiled at 95°C for 5 min and placed in 10% SDS-polyacrylamide gel for electrophoresis. The protein images from the gel were transferred to a nitrocellulose membrane and blocked with 5% nonfat milk in PBST solution (phosphate buffer saline plus 0.1% Triton X-100). After being washed twice with PBST, the membrane was incubated with 1:1,000 dilution of antibodies (anti-Gp96, NeoMarkers) at room temperature for 2 h. The membrane was washed again and incubated again with anti-mouse immunoglobulin G antibody conjugated with horseradish peroxidase. The level of expression was quantified by densitometry using a FluorChem Image System (Cell Kinetics Inc.).

Table 1. Candidate genes up-regulated in radioresistant nasopharyngeal carcinoma cells

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<th>Annotation</th>
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<td>Gp96</td>
<td>Stress response, chaperone activity</td>
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<tr>
<td>U88323</td>
<td>GDF-15</td>
<td>Apoptosis, cytokine activity</td>
<td>3.12 ± 0.55</td>
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<td>AF055001</td>
<td>HERPUD1</td>
<td>Response to ER stress, UV irradiation</td>
<td>2.79 ± 1.18</td>
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<tr>
<td>M15330</td>
<td>IL-1, β</td>
<td>Inflammation, cell proliferation</td>
<td>2.63 ± 0.79</td>
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<tr>
<td>BC009951</td>
<td>Cullin 2</td>
<td>Cell cycle</td>
<td>2.22 ± 0.41</td>
</tr>
<tr>
<td>BC011757</td>
<td>GADD45A</td>
<td>DNA repair</td>
<td>2.11 ± 0.27</td>
</tr>
<tr>
<td>M77693</td>
<td>SAT</td>
<td>Acetyltransferase activity</td>
<td>2.02 ± 0.05</td>
</tr>
</tbody>
</table>
peroxidase. Membranes were treated with enhanced chemiluminescence developing solution (Amersham Pharmacia Biotech) and exposed to X-ray film. Actin expression was used as an internal control.

**Cloning of siRNA Plasmids**

The pTOPO-U6 vector was used to construct siRNA plasmids as previously described (16). A 22-nt sense and antisense hairpin oligonucleotide was generated that was complementary to either gp96 or GDF15 mRNA. The sequences of these specific nucleotides in the hairpin structures are gp96si: 5'-GGAGAUAUGGUGCCAGAAA-3'; GDF15-si: 5'-GGAGAUCUGGUAACGAGG-3'; GDF15-si-1: 5'-GGAGAUCUCACGCAGAAGUGCG-3'. This hairpin oligonucleotide included two restriction enzyme cleavage sites corresponding to the blunt end and overhang end matching EcoRV- and BsoI-digested pTOPO-U6. Two siRNA sequences of each gene were designed, named gp96si1 and gp96si1-1, and GDFsi and GDFsi-1 (Fig. 3A-1 and B-1). Ligation between the hairpin oligonucleotides and pTOPO-U6 at these cloning sites produced the specific siRNA plasmid.

**Plasmid Transfection**

For plasmid transfection, cells were seeded at a density of 5 × 10^5 in a 100-mm dish and cultured for 16 h. When 60% confluence was reached, the cells were transfected with 6 µg of siRNA plasmids or the vector plasmids using LipofectAMINE 2000 reagent (Invitrogen) in OptiMEM medium (Invitrogen) for 16 h; after which, the medium was replaced with fresh complete medium. The transfection efficiency was ~70% for both NPC cell lines.

**Cell Growth Analysis in Response to Irradiation**

To determine whether irradiation induced a delay in cell growth, 0.5 × 10^4 NPC cells were plated into a 24-well cell culture plate. After incubation for 24 h, the cells were irradiated with various doses (4-12 Gy). Cell growth was monitored by counting cell numbers at various time intervals.

**Clonogenic Survival Assay**

For clonogenic assays, cells were plated onto a six-well plate and were exposed to a range of radiation doses (4-12 Gy). After irradiation, the cells were cultured for 10 days, and the number of surviving colonies (defined as a colony with ≥50 cells) was counted. The survival fraction was calculated as the numbers of colonies divided by the numbers of cells seeded times plating efficiency. Plating efficiency was calculated as the numbers of colonies minus the numbers of cells plated times 100.

**Flow Cytometry Analysis**

Cells were trypsinized and analyzed during exponential growth. The cell pellets were fixed with ice-cold 70% ethyl alcohol in PBS at −20°C for 1 h and then centrifuged at 1,500 rpm for 5 min. The pellets were suspended and incubated with 0.5% Triton X-100 (Sigma Chemical Co.) and 0.05% RNase (Sigma) in 1 mL PBS at 37°C for 30 min and then centrifuged at 1,500 rpm for 5 min. The cell pellets were resuspended and incubated with 40 mg/mL propidium iodide (PI) in 1 mL PBS at room temperature for 30 min. Samples were immediately analyzed by a FACScan flow cytometry (Becton Dickinson). The distribution of cell cycle phases was determined using Cell Quest Pro and ModFit software. Two independent experiments were done for each data set.

**Results**

**Establishment and Authentication of Radioresistant Subclone Cells**

For each cell line, the sublethal radiation dose was determined by dose titration. Nearly all NPC-076 cells were killed after 5 days of irradiation at a dose of 12 Gy, and no cells were recovered at doses higher than 12 Gy. A dose of 11 Gy was therefore considered sublethal and used for the selection of NPC-076–radioresistant subclones. Similarly, the sublethal dose for the selection of NPC-BM1–radioresistant cells was determined to be 10 Gy. The first generation of subclone cells was generated from the culture of the surviving fraction of the parental cells irradiated with the respective sublethal doses. Similarly, the second generation of subclone cells was generated from the culture of the surviving fraction of the first-generation cells irradiated with the respective sublethal doses. Finally, the fourth generation of NPC-076– and BM1-radioresistant subclones were established and designated as 076-radioresistant and BM1-radioresistant cells.

The NPC-radioresistant subclones were subjected to irradiation to authenticate that they were radioresistant. The cell growth status of the parental, the second-generation, and the radioresistant subline cells in response to the sublethal dose of irradiation was monitored. Two independent experiments were done; the representative results were shown in Fig. 1A and B, respectively, for 076 and BM1 cells. As shown, the parental cells gradually died with time; cell death was delayed in the second-generation cells, in contrast to the survival of radioresistant subclones. Apparently, the radioresistant subclones were more resistant to irradiation.

To further verify the radioresistant phenotypes of radioresistant subclones, BM1-radioresistant subclone was further examined by clonogenic survival assay. The NPC cells were irradiated with various dose (0-12 Gy) and incubated for 12 days to allow cell colony formation. As shown in the Fig. 1C, the surviving colonies of the radioresistant cells were significantly more and bigger than the parental cells. The colonies of the 8-12-Gy–treated cells were further quantified and shown in Fig. 1D. At a dose of 8 Gy, the differential number of the survival colony between parental and radioresistant cell was most significant (P = 0.008). Furthermore, the BM1-radioresistant subclones were subjected to a low dose of radiation to examine the effect on cell growth. As shown in the Fig. 1E, irradiation with 4 Gy delayed growth in both parental and radioresistant cells, but the radioresistant subclone grew faster than the parental cells. The difference between irradiated parental and radioresistant cells was statistically significant after 96 h of
irradiation. This finding is consistent with the reported typical radioresistant phenotype (17), indicating successful subcloning of radioresistant cells for these two NPC cell lines.

**Screening for Radioresistance Genes by cDNA Microarray**

Both 076-radioresistant and BM1-radioresistant subclones, along with their corresponding parental cells, were...
subjected to cDNA microarray analysis. The microarray chips included 7,334 genes, of which 3,352 were detected in 076 cells and 3,610 were detected in BM1 cells. Up-regulation was defined as a gene expression that was 2-fold higher in the subclone (log2 ratio >1), and down-regulation was a 2-fold lower expression in the subclone (log2 ratio <1). In the microarray analysis of the BM1-radioresistant cells, 13 genes were up-regulated, and 16 were down-regulated, whereas 17 genes were up-regulated, and 10 were down-regulated in 076-radioresistant cells. Of the up-regulated genes, seven genes were shown highly differentially expressed in the radioresistant subclones, including gp96, GDF15, HERPUD1, IL1-β, cullin 2, GADD45A, and SAT (Table 1). These results suggest that these genes may contribute to the radioresistant phenotype.

**gp96 and GDF-15 Up-regulated in NPC-Radioresistant Cells**

To assess a biologically meaningful function of putative radioresistant genes, we further investigated the two genes with the greatest degree of up-regulation, gp96 and GDF15. RT-PCR analysis was first done in parental and radioresistant subclone cells to confirm the results of microarray screening. As shown in Fig. 2, gp96 and GDF-15 were up-regulated in both 076-radioresistant and BM1-radioresistant cells compared with the corresponding parental cells. Gp96 was overexpressed, with 1.52- and 2.06-fold, respectively, for 076 and BM1 cells. GDF-15 was overexpressed, with 1.70- and 1.89-fold, respectively, for NPC-076 and BM1 cells. This confirmation showed that these two genes were worth assessing for a possible role in the radioresistant phenotype.

**gp96 or GDF-15 Knockdown Increases Radiosensitivity in NPC Cells**

To assess the radioresistant effects of gp96 and GDF-15 in NPC cells, specific gene expression was knocked down by RNA interference. For each gene, two clones of siRNA plasmids (si and si-1) were designed (Fig. 3A-1 and B-1). Gene expression was examined by either Western blot (gp96) or RT-PCR (GDF-15) after transfection of the plasmids into BM1 cells for 2 days. The two gp96 siRNA clones had different levels of RNA inhibition, with minimal inhibition by gp96si-1 but high levels (93%) of inhibition with gp96si (Fig. 3A); thus, gp96si was used for further study. Both GDFsi and GDFsi-1 strongly inhibited (>90%) GDF-15 expression (Fig. 3B); and GDFsi was chosen for further experiments.

BM1 cells were transfected with plasmids containing gp96i, GDFsi, or vector as a control. Cell growth was then monitored for up to 6 days with and without 5 Gy of radiation. Figure 4A and B shows the results of cell growth after gp96 and GDF15 knockdown. As shown, in the absence of radiation, the growth rate of gp96 or GDF15 knockdown cells was similar to that of control cells. However, when the cells were irradiated, the gp96 knockdown cells grew at a much slower rate than the control cells, with 26.2% less than controls at day 4 and 31.8% less at day 6 (Fig. 4A). Knockdown with GDFsi had a

![Figure 2](image2.png)

**Figure 2.** Differential expression of gp96 and GDF-15 between nasopharyngeal carcinoma parental (PT) and radioresistant subclone (076 and BM1) cells by RT-PCR. The expression of β-actin gene was used as an internal control for each gene. The relative density in each sample was indicated at the bottom of the band after normalization with β-actin level in each individual sample.

![Figure 3](image3.png)

**Figure 3.** Demonstration of siRNA effect. Two clones of siRNA plasmids (si and si-1) for gp96 and GDF-15 were constructed. These plasmids were transfected into nasopharyngeal carcinoma BM1 cells for 2 days, and the effect on gene expression was examined. A, Western blot analysis shows better inhibition by gp96si than by gp96si-1. B, RT-PCR analysis shows an excellent inhibition by both GDF15si and GDF15si-1. The relative density in each sample was indicated at the bottom of the band after normalization with the β-actin level in each individual sample.
similar effect, with 32.4% and 41.4% inhibition of growth compared with control cells at days 4 and 6, respectively (Fig. 4B). These results indicate that knockdown of either gp96 or GDF15 increased the radiosensitivity of BM1 cells.

A colonogenic survival assay was also done to examine the effects of cell killing by irradiation after knockdown of gp96 or GDF15 expression. BM1 cells were transfected with either of the two specific siRNA (gp96si or GDFsi) or with nonspecific siRNA (Luci-si) or vector plasmid as controls. The cells were treated with various radiation doses (0-10 Gy) and were then continuously cultured with colony formation. Both gp96 (Fig. 5A) and GDFsi (Fig. 5B) knockdown resulted in fewer surviving colonies compared with cells transfected with either of the two control plasmids. The lower survival was statistically significant for both gp96si ($P < 0.01$ at 6 Gy and $P < 0.001$ at 8 and 10 Gy) and GDFsi ($P < 0.01$ at 6 and 8 Gy and $P < 0.001$ at 10 Gy) compared with the control cells. These results further show that knockdown of these two genes induces radiosensitization.

Radiosensitization Induced by gp96 or GDF-15 Knockdown Is Associated with G2-M Arrest

To further assess cell proliferation mechanism associated with siRNA-induced radiosensitization, flow cytometry was used to examine the cell cycle distribution in gp96si- or GDFsi-transfected cells. Before irradiation, BM1 cells were arrested in the G0 phase by serum-starved culture. After irradiation with 5 Gy, the cell cycle distribution was recorded at various time intervals up to 40 h. Two independent experiments were done, and both showed the same trend, with a represent result shown in Figure 6. Compared with Luci-si transfectant controls, an increase in G2-M phase cells was observed in both gp96si (Fig. 6A) and GDFsi (Fig. 6B) transfectants after 24 h of irradiation. On average, the percentage of gp96 knockdown cells in the G2-M phase increased by 49%, and that of GDF15 increased by 39% after 24 h. This increment of the G2-M phase cells can sustain up to 40 h and gradually decrease. As shown in Figure 6, for example, the percentage of gp96 knockdown cells in the G2-M phase increased by 73% at 24 h (from 18.2% to 31.4% of total cells) and still maintained by 41% at 40 h (from 15.0% to 21.3%; Fig. 6A). The percentage of GDF-15 knockdown cells in the G2-M phase increased by 34% at 24 h (from 18.2% to 24.4% of total cells) and was still maintained by 44% at 40 h (from 15.0% to 21.7%; Fig. 6B). Because there is no observable cell death fraction (sub-G1 phase cells) was found during flow-cytometric analysis, and the inhibition of gene expressions in siRNA transfectants was gradually diminished.
after 36 h, this temporary effect of G2-M arrest may be due to the siRNA breakdown in the cells. In all, these results indicate that the radiosensitive phenotype induced by gp96 or GDF-15 knockdown is associated with an increase in G2-M arrest.

**Discussion**

In this investigation of two NPC-radioresistant cell lines established by sublethal doses of radiation, we identified seven genes that were up-regulated in both radioresistant subclones compared with the parental cells from which the

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### Table A

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**Figure 6.** Alteration of cell cycle distribution after siRNA transfection into nasopharyngeal carcinoma cells in response to irradiation. Before irradiation, cells (0.5 × 10⁵ cells per well in 10-well plate) were transfected for 24 h with either gp96si (A) or GDFsi (B), along with the control plasmids (Luci-si). The cells were then synchronized to G0 phase by replacing the culture medium with serum-free medium. The transfectants were treated with a single dose of 5 Gy of irradiation and continuously cultured for up to 40 h. In each sample, the cell cycle distribution was determined by flow cytometry analysis. Arrows, location of G2-M phase in the cell cycle illustration.
subclones were derived. Of these seven, two genes, gp96 and GDF15, were strongly overexpressed (Fig. 2). We were then able to show that transfection of siRNA in plasmids to knock down each of these genes rendered the NPC subclones more sensitive to radiation.

Gp96 (also known as GRP94) was first identified and reported as a glucose-regulated protein that responds to hypoglycemic stress (18). Gp96 is an endoplasmic reticulum chaperon, belonging to heat shock protein 90 (HSP90) family. It assists in the folding and export of soluble and membrane-bound oligomeric proteins, including immunoglobulins (19), epidermal growth factor receptor (20), Toll-like receptor, and integrins (21). It is also able to elicit various immune responses, including the generation of antigen-processing peptides suitable for the assembly of MHC class I molecules (22), and to elicit antitumor immune response (23). A recent study using a mouse model has shown that gp96-based immunotherapy increases the efficacy of radiotherapy (24). In the present study, we found that knockdown of the gp96 gene with siRNA in NPC-radioresistant cells resulted in growth delay and a reduction of clonogenic survival in response to radiation (Figs. 4A and 5A). These results were in agreement with previous findings that combined the inhibition of signal-regulated kinases and HSP90-sensitized colon carcinoma cells to ionizing radiation (25). HSP90 has also been reported to modulate Akt kinase activity (26) and to compromise the DNA damage in response to radiation (27). Because gp96 is a member of the HSP90 family, whether Akt is also a client molecule of gp96 chaperon leading to cytoprotection and increase of cell survival is awaiting to be investigated.

In the same way, we found that GDF-15 was similarly involved in radiosensitization, an effect that could be inhibited by knockdown of the gene. This gene has various names, including MIC-1, PLAB, PTGFβ, PDF, and HP00269. It was discovered and cloned almost simultaneously by different groups in 1997 (28–31). GDF-15 is a member of the transforming growth factor β (TGFβ) superfamily, with 20% to 25% homogeneity to TGFβ1-3 and 30% to 38% to bone morphogenetic proteins (28–31). GDF-15 is an important downstream mediator of the response to DNA damage and is a transcriptional target of p53 (32). Overexpression of GDF-15 leads to the induction of p21 and increased G1 cell cycle arrest and apoptosis (33). Other investigators, however, have shown a contrasting function of GDF-15 in preventing hypokalemic-induced cell death in cerebellar granule neurons and preventing the formation of radical oxygen species (34). In the present study, we found that knockdown GDF-15 in NPC cells resulted in growth delay and a reduction of clonogenic survival in response to radiation (Figs. 4B and 5B). Our results are more consistent with later findings of GDF-15 in the role of cytoprotection. Whether this molecule also participates in the mechanism of DNA damage repair or whether its function is p53 associated will be further investigated.

Tumor cells are known to be most sensitive to radiation-induced cell death when synchronized in the G2–M phase of the cell cycle, as the DNA is more vulnerable to radiation-induced damage during mitosis (35). We observed that the gp96 and GDF15 knockdown cells had increased G2-M arrest in response to irradiation (Fig. 6A and B). This may explain how knockdown induces radiosensitivity. Previous studies have shown several key effectors to be involved in radiation-induced DNA damage and the G2-M cell cycle arrest. Inactivation of Cdc25 and activation of Wee1 can lead to disassembly of Cdc2-cyclin B heterodimers, arresting cells in the G2-M phase (35). In addition, spindle disassembly caused by depolymerization of microtubules also triggers arrest of cells in this phase (36). Whether gp96 and GDF15 are also involved in these cellular mechanisms remains to be elucidated.

In conclusion, we have identified at least seven genes apparently associated with the radioresistant phenotype in NPC cells, including gp96 and GDF15. Knockdown of gp96 or GDF15 expression results in the radiosensitization of NPC cells. This study thereby contributes basic information about some of the genes involved in radioresistance. This may eventually contribute to clinical investigation as prediction markers, prognostic factors, as well the development of radiosensitizing molecules for therapeutic use.

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