Research Article

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GADD45B induced the enhancing of cell viability and proliferation in radiotherapy and increased the radioresistance of HONE1 cells

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Abstract: This study aimed to investigate the key role and mechanism of GADD45B in the radiation resistance of nasopharyngeal carcinoma (NPC) cell lines. Radiotherapy-resistant HONE1 (HONE1-R) cells with stable genetic radioresistance were cultured under continuous radiation stimulation. CCK-8 and clone formation assays were used to verify the radioresistance of the cell line. Transcriptome sequencing was used to identify the most important differential signaling pathway in the cell line. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) and Western blot analysis were used to verify the sequencing results. GADD45B-siRNA was used to knock down the key gene so as to verify the downstream gene expression and analyze its mechanism. The transcriptome analysis showed that 702 genes were upregulated and 772 genes were downregulated in the HONE1-R cell lines. The core differential signaling pathway was mitogen-activated protein kinase (MAPK) signaling pathway, and the core differential gene was GADD45B.

Keywords: GADD45B, MAPK signaling pathway, nasopharyngeal carcinoma, radiation resistance, cell viability, proliferation

1 Introduction

Nasopharyngeal carcinoma (NPC) is a malignant tumor of the head and neck originating from the epithelial cells of the nasopharynx. Squamous cell carcinoma is the main pathological type of NPC. Comprehensive treatment based on radiotherapy is preferred for patients with NPC due to the complexity of anatomical location and high sensitivity to radiation [1]. Early-stage NPC can be cured by radiotherapy, and the 5 year survival is up to 90% [2]. Nevertheless, more than 70% of patients with NPC are in the middle- or advanced-stage disease at initial diagnosis. The 5 year survival of patients with Stages III and IV NPC is only 53–81.8 and 28–66.39%, respectively [3,4]. Local recurrence and distant metastases of NPC caused by radiotherapy resistance are the major obstacles in the clinical treatment, despite some progress in the current therapeutic strategies [5,6]. Secondary radiotherapy protects against radiotherapy resistance or recurrence of NPC, but easily leads to severe complications and yields limited benefits [7]. Therefore, clarifying the molecular mechanism underlying radiotherapy resistance of NPC and enhancing radiotherapy sensitivity are important for improving the clinical outcomes.

The transcriptome is a set of all RNA transcripts. Transcriptome analysis of a specific tissue or cell at a
specific stage or in a functional state from an overall perspective can clarify the molecular mechanism of diseases [8]. This analysis has been applied to the research on NPC, achieving a certain outcome. Zhou et al. [9] identified 420 differentially expressed long noncoding RNAs and 31 circular RNAs in the transcriptome information of NPC and predicted their targets. Chang et al. [10] analyzed the transcriptome data of NPC tissues. They found that SSX2IP was significantly upregulated in NPC tissues, which was closely linked to the invasive ability of NPC cells and predicted a poor prognosis. However, the transcriptome analysis of radiotherapy-resistant NPC cells has been rarely reported.

In this study, a stable NPC cell line, radiotherapy-resistant HONE1 (HONE1-R), was first established by comparing the transcriptome sequencing data of HONE1-R and sensitive cell lines. Then, the study focused on the key gene GADD45B related to NPC and its related signaling pathway. The mechanism of the radioresistance of GADD45B to HONE1-R cell line was further explored by knocking down the expression of GADD45B.

## 2 Methods

### 2.1 Cell culture

The NPC cell line HONE1 was purchased from the Query Network for Microbial Species of China (http://www.biobw.org; catalog number: bio-105809). The cells were cultivated in the dulbecco’s modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin in a humidified atmosphere of 5% CO₂ at 37°C. Adherent cells with more than 80% confluence were passaged using trypsin.

### 2.2 Generation of the HONE1-R cell line

HONE1 cells in the logarithmic growth phase were digested into a single-cell suspension and implanted in a six-well plate (4 × 10⁶ cells per well). After adherence, the cells were induced with 8 Gy irradiation. Surviving subclonal cells were reinduced with 6 Gy irradiation, cultured, and passaged for live cells for a total of three periods. Finally, the surviving cells were induced with 8 Gy irradiation, and the live cells were stable HONE1-R cells. The HONE1-R cells were subcultured to more than five generations and stably inherited.

### 2.3 CCK-8 assay

HONE1 and HONE1-R cells in the logarithmic growth phase were used to prepare a cell suspension (2 × 10⁵ cells/100 µL), which was implanted in a 96-well plate. After cell adherence, 6 Gy irradiation was used. In each well, 10 µL of CCK-8 solution (Apexbio, USA) was added at the indicated time points. After 1 h, the optical density at 450 nm was measured using a microplate reader to calculate the proliferation rate.

### 2.4 Colony formation assay

The HONE1 and HONE1-R cells were implanted in a six-well plate (300 cells per well). Until cell adherence, the cells were irradiated with 6 Gy X-ray radiation and cultured until the formation of visible colonies. They were fixed in methanol for 15 min, washed with phosphate-buffered saline, and stained with 1% crystal violet for 20 min. Visible colonies containing more than 50 cells were captured and counted.

### 2.5 Construction of a specific cDNA library and high-throughput sequencing

Cellular RNAs were isolated using TRIzol and subjected to agarose gel electrophoresis and purification. The mRNA was enriched using oligomeric dT nucleotide-coated resins and separated into short reads by adding the fragmentation buffer. Using the mRNA as a primer, the first strand of cDNA was synthesized through Random Primer 6; the second strand of cDNA was then synthesized by the induction of the loading buffer, deoxyribonucleotide triphosphate, DNA polymerase I, and RNaseH. Double-stranded cDNA was purified using AMPure XP beads, followed by the generation of a poly-A tail and the connection of DNA ligase. Subsequently, optimal-sized segments were selected using AMPure XP beads. The second-strand cDNA containing U was degraded by the USER enzyme to ensure that all sequencing information was from the first-strand cDNA. Finally, cDNA was subjected to polymerase chain reaction (PCR), and purified PCR products were used to generate a cDNA library.

The cDNA library was primarily quantified using Qubit 3.0. The insert size of the cDNA library was detected using Qsep100. The qualified library was subjected to sequencing using Illumina HiSeqTM2000/Miseq, and the obtained clean reads were compared with the ribosome database to clear the ribosome sequences.
2.6 Analysis of differentially expressed genes

The posterior distributions (Z) of reads per million mapped reads calculated by the Bayesian method were analyzed using generalized fold change (GFOLD), and GFOLD (Z) was calculated. A differentially expressed gene was determined when its GFOLD (Z) was >0 or <0. The fold change was also calculated. A gene showing a log₂ FC ≥1 or ≤−1 was considered as a differentially expressed gene, and a gene showing a false discovery rate of ≤0.05 was considered as a significantly differentially expressed gene. The functional annotation chart was produced using DAVID (https://david.ncifcrf.gov/).

2.7 Bioinformatics analysis

The gene ontology (GO) terms classified gene functions into molecular functions (MF), biological processes (BP), and cellular components (CC). GO annotations were obtained using the database. A P value of <0.05 was considered as a cut-off value, and statistically significant annotations were analyzed using the cluster profiler package.

Target genes in different pathways were analyzed using Kyoto Encyclopedia of Genes and Genomes (KEGG) with the cut-off value at P < 0.05, and they were later assessed using the cluster profiler package.

The signaling pathway networks were depicted using Cytoscape 3.4.0 software (Institute for Systems Biology). Each pathway network was depicted based on the pathway terms, and the networks with P < 0.05 were analyzed using KEGG.

2.8 qRT-PCR

Ten differentially expressed genes were subjected to qRT-PCR. The primer sequences are listed in Table 1. Reverse transcription was conducted using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara Biotechnology Co., Ltd); the(122,322),(638,915)

| Genes     | Sequences                                |
|-----------|------------------------------------------|
| CXCL10-F  | 5'-GACTCTGAGTGGAACTCAAGGAT-3'           |
| CXCL10-R  | 5'-GTGGCAATGCTCACAACAGC-3'              |
| IKBKGF    | 5'-GAGCTCCAGAGATGCCATC-3'               |
| IKBKGR    | 5'-CTCAGATCTGCTGAGTCG-3'                |
| MAP4K2-F  | 5'-GAGGCCGGCCAAAGAGGAGA-3'              |
| MAP4K2-R  | 5'-GTAGTCGAAGAGCAGATC-3'                |
| MAPKAPK3-F| 5'-CTACACTCCCCATATTGT-3'                |
| MAPKAPK3-R| 5'-TGACCACCTGGATAG-3'                   |
| Akt3-F    | 5'-AATCTGAAGCTCCCAAACG-3'               |
| Akt3-R    | 5'-ACACTCCAGCGTCAAGAGG-3'               |
| GADD45B-F | 5'-TGACAACGACATCAACATC-3'               |
| GADD45B-R | 5'-GTGACCAAGAGCAATGACG-3'               |
| JUN-F     | 5'-AACTGATGACCACTGGATACAG-3'            |
| JUN-R     | 5'-AATGTTGTCACAGTGTCC-3'                |
| FOS-F     | 5'-AGACAGGACGAAAATGAGG-3'               |
| FOS-R     | 5'-AGCTTTTCTCCCTCTCTTTTGG-3'            |
| MMP9-F    | 5'-TACACTGGAGAGAATGACG-3'               |
| MMP9-R    | 5'-AGGGGGAGGACATGAG-3'                  |
| TNFSF10-F | 5'-GTGGAAAAATAGGAGACG-3'                |
| TNFSF10-R | 5'-GAAGCTGTCGATAGGAGAAC-3'              |
| β-Actin-F | 5'-ATTGCCGGAGGGAGCAT-3'                 |
| β-Actin-R | 5'-CAAGATCATTGGCTCCCTGAGC-3'            |

2.9 Western blot analysis

Total proteins collected from the HONE1 and HONE1-R cells were subjected to concentration determination by the bicinchoninic acid method. All the antibodies were purchased from Abclonal (Wuhan, China) or Abcam (Cambridge, UK); the catalog numbers were as listed: CXCL10 (A1457, Abclonal), IKBKGF (A12536, Abclonal), MAP4K2 (A17524, Abclonal), MAPKAPK3 (A18095, Abclonal), AKT3 (A12909, Abclonal), GADD45B (ab205252, Abcam), JUN (A16905, Abclonal), FOS (A17351, Abclonal), MMP9 (A2095, Abclonal), TNFSF10 (A2138, Abclonal), β-actin (AC038, Abclonal), and the secondary antibody (AS014, Abclonal). Nonspecific antigens were blocked in skimmed milk for 1 h. The membranes were immunoblotted with primary antibodies (1:1,000) at 4°C overnight and secondary antibodies (1:1,000) at room temperature for 1 h. Band exposure was achieved using the enhanced chemiluminescence method, and ImageJ software was used for the semiquantitative analysis of proteins.

2.10 TCGA database analysis

The GADD45B expression level in cancer and paracancer tissues was searched from TCGA (the Cancer Genome...
Atlas) database. The tumor type was squamous cell carcinoma of head and neck. After downloading all the relevant data, 43 pairs of paired tumor tissue and adjacent tissue data were obtained. With the same method, 43 pairs of paired data were obtained. \( \log_2(\text{count} + 1) \) was used to analyze the gene expression in these samples.

2.11 Cell transfection

The si-GADD45B and the negative control (si-NC) oligonucleotide sequences in this study were synthesized by biological engineering. The sequences were as follows: si-GADD45B-1: sense: r(CGUUCUGCUCGACAAUGA)dTdT; antisense: r(UCAUUGUGCAGCAGAAG)dAdT; si-GADD45B-2: sense: CGACAACGCGGUUCAGAAGU; antisense: 5'-CUUC UGAACCCGGUGUGGUU-3'. The cells were grown to more than 80% confluence and then switched to a serum-free medium for 4 h. The transfection plasmid gently mixed with polyethylenimine was then applied and incubated at room temperature for 40 min. Subsequently, the reduced serum medium (Opti-MEM) was replaced for 24 h of cell culture, followed by conventional culture in the DMEM containing 10% FBS and 1% penicillin and streptomycin.

2.12 Statistical analysis

Statistical analyses were performed using GraphPad Prism 8. Data were expressed as mean ± standard deviation (mean ± SD). All data conformed to normal distribution. Comparisons among multiple groups were analyzed using one-way analysis of variance, followed by Tukey's or Bonferroni's post hoc test. A \( P \) value of <0.05 indicated a statistically significant difference. All experiments were performed in triplicate.

Figure 1: Verification of the ability of radiotherapy resistance of HONE1-R cell lines: (a) cell viability of HONE1 and HONE1-R cell lines treated with 6 Gy and (b) cell proliferation of HONE1 and HONE1-R cell lines treated with 6 Gy.
3 Results

3.1 Verification of the ability of radiotherapy resistance of HONE1-R cell lines

The results showed that the cell viability of HONE1 cell lines decreased by 82.64%, whereas the cell viability of HONE1-R cell lines decreased by 28.09% (Figure 1a). The colony formation number of HONE1 cell lines decreased by 79.9%, and the colony formation number of HONE1-R cell lines decreased by 32.1% (Figure 1b). The results showed that the 50% inhibition dose for HONE1 and HONE1-R cell viability was 1.70 and 6.80 Gy, respectively (Figure 2a), and the 50% inhibition dose for colony formation was 2.09 and 7.61 Gy, respectively (Figure 2b). The findings suggested that the HONE1-R cell line had strong radioresistance and stable heredity.

Figure 2: Determination of IC₅₀ on cell viability and proliferation of HONE1 and HONE1-R cells: (a) IC₅₀ of radiation suppression on cell viability and (b) IC₅₀ of radiation suppression on cell proliferation.
3.2 Mitogen-activated protein kinase (MAPK) was the most important signaling pathway in HONE1-R radiotherapy resistance

Transcriptome sequencing of HONE1 and HONE1-R cell lines showed that 702 genes were upregulated and 772 genes were downregulated in the HONE1-R cell lines (Figure 3a). GO analysis showed that the function of differentially expressed genes mainly concentrated in protein heterodifferentiation activity (MF), cell–cell junction (CC), protein extracted matrix (CC), and regulation of myoid cell differentiation (BP) (Figure 3b). KEGG analysis showed 18 differential signaling pathways; the most significant differential pathways included systemic lupus erythematosus, astronomy, interleukin 17 signaling pathway, apoptosis, tumor necrosis factor (TNF) signaling pathway, and MAPK signaling pathway (Figure 3c). The analysis results of differential signaling pathway interaction network are shown in Figure 3d. The most central network was the MAPK signaling pathway; in addition, the apoptosis pathway and TNF signaling pathway were also important.

3.3 GADD45B might be a key gene in the HONE1-R radiotherapy resistance

Ten differentially expressed genes between HONE1-R and HONE1 cells were selected from MAPK, apoptosis, and TNF pathways to verify the results of sequencing; further validation was performed using qRT-PCR and Western

![Image](image_url)
blot analysis. Among these 10 genes, IKBKG, JUN, and AKT3 were significantly changed in MAPK, apoptosis, and TNF signaling pathways; the other 6 genes were key genes in these three pathways. The results were compared with the sequencing results and were consistent with the sequencing data. The protein levels also showed the same trend as sequencing (Figure 4a and b). GADD45B mRNA and protein expression levels were significantly upregulated, suggesting that GADD45B might be the key gene of HONE1-R radiation resistance.

### 3.4 GADD45B effectively improved the activity and proliferation of HONE1-R cells under radiation

The GADD45B expression level was significantly downregulated in squamous cell carcinoma of head and neck (Figure 5a). After transfection of GADD45B-siRNA1 and GADD45B-siRNA2, the expression level of GADD45B in HONE1-R cells was significantly downregulated, showing good transfection efficiency and effectiveness (Figure 5b). Subsequently, HONE1-R cells transfected with si-NC and GADD45B-siRNA1 were exposed to 6 Gy radiation, and the cell viability was detected. The results showed that the cell viability of HONE1-R cells was significantly inhibited after the inhibition of GADD45B (Figure 5c). The clone formation results showed that the HONE1-R cell line transfected with GADD45B-siRNA1 also had weaker proliferation ability and radioresistance (Figure 5d). Subsequently, the downstream gene of GADD45B was detected. The results showed that after exposure to 6 Gy irradiation, the levels of cyclinB1 and p-CDK-1 in HONE1 cells transfected with GADD45B-siRNA1 decreased abnormally compared with that transfected si-NC (Figure 6a and b). These results suggested that GADD45B could effectively improve the cell viability and proliferation ability of HONE1-R cells under radiation, thus significantly improving the radiation resistance ability of the HONE1-R cell line.

### 4 Discussion

Currently, a comprehensive strategy based on chemotherapy is the most common option for patients with NPC [1]. Unfortunately, the development of radiotherapy resistance leads to treatment failure and poor prognosis owing to local recurrence or distant metastases [6]. Hence, the relevant genes and signaling pathways involved in radiotherapy resistance of NPC were analyzed in this study to improve the therapeutic efficacy and prognosis. By generating the HONE1-R cell line and analyzing the sequencing data in HONE1-R and HONE1 cells, the radiotherapy-resistant genes in HONE1-R cells were found to be mainly enriched in MAPK, apoptosis, and TNF signaling pathways, MAPK is the most significantly different signaling pathway between HONE1-R and HONE1 cell lines; GADD45B might be an important gene in HONE1-R radiotherapy resistance.

MAPKs are serine/threonine protein kinases widely present in cells. An abnormally activated MAPK signaling pathway causes the loss of differentiation and apoptosis capacities, uncontrolled proliferation, and development of drug resistance in cancer cells [11]. Williams et al. [12] demonstrated that the application of the MAPK inhibitor

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PD0325901 significantly enhanced the radiotherapy sensitivity of pancreatic cancer cells, and combined inhibition of MAPK and Protein kinase B further enhanced the sensitivity. Sun et al. [13] suggested that the overexpression of TOB1 elevated the radiotherapy sensitivity of breast cancer cells and cervical cancer cells by activating the MAPK/extracellular regulated protein kinases signaling pathway and regulating p53 phosphorylation. He et al. [14] generated ATM-deficient bladder cancer cells highly sensitive to irradiation, and the MAPK and nuclear factor kappa-B (NF-κB) signaling pathways were significantly inactivated in these cells. Yu et al. [15] conducted high-throughput sequencing in irradiated HeLa and control cells. MAPK signaling, endocytosis, axon guidance, neurotrophic...
signaling, and soluble N-ethylmaleimide sensitive-factor attachment receptor interactions in vesicle trafficking were found to be significantly different between these cells. Cook et al. [16] proved that Cox-2-derived PGE2 induces Id1 via EP4-dependent activation of MAPK signaling and the Egr1 transcription factor in glioblastoma cells. PGE2-mediated induction of Id1 was required for optimal tumor cell self-renewal and radiation resistance, and finally mediated radiation resistance in patients with glioblastoma. These previous findings suggested that the MAPK signaling pathway might play an important role in tumor radiation tolerance. The KEGG and signaling pathway network analysis showed that the MAPK signaling pathway was the most significant and the most core signaling pathway between HONE1-R and HONE1 cell lines. The MAPK signaling pathway was abnormally activated in the HONE1-R cell line: 18 genes were upregulated and 14 genes were downregulated. Sequencing, RT-qPCR, and Western blot analysis confirmed that GADD45B, AKT3, JUN, and FOS were significantly upregulated and IKBKGN, MAP4K2, and MAPKAPK3 were significantly downregulated in the MAPK signaling pathway, indicating their important role in radiation resistance of the HONE1 cell line.

The GADD45 family includes GADD45α, GADD45β, and GADD45γ, which are related to stress signal and regulate cell cycle, proliferation, differentiation, survival, senescence, and apoptosis [17,18]. GADD45B is widely distributed in various mammalian tissues. The expression level of GADD45B is low in the normal physiological state, but it increases significantly under the influence of environmental stress or injury stimulation [19]; GADD45B has different expression and regulation mechanisms in different cell states, and plays different or even opposite roles [20]. Previous studies confirmed that the regulatory effect of GADD45B on cell cycle mainly depended on the MAPK signaling pathway. GADD45B can directly bind to MEKK4 and MEKK7 in the MAPK signaling pathway and inhibit the JNK/p38 signaling pathway [21]. For example, Cho et al. [22] proved that the expression of GADD45B increased under the hypertonic condition and mediated G2/M-phase arrest of the cell cycle, which promoted DNA repair and cell proliferation. Yu et al. [23] confirmed that the activation of NF-κB also increased the expression of GADD45B, inhibited the function of GADD45α, increased the degradation of p53, and thus inhibited apoptosis, suggesting that the balance of GADD45α/GADD45B might determine the survival or apoptosis of cells. Recent studies have shown that GADD45B is crucial in tumor radiotherapy. Barros-Filho et al. [24] found that the increased expression of GADD45B was an important marker of shortened disease-free survival in patients with papillary thyroid cancer after total thyroidectomy and radioiodine therapy. Vairapandi et al. [25] studied lung cancer cell lines. The results showed that GADD45B caused G2/M cell cycle arrest by acting on CDK1/cyclin B1. However, GADD45B could activate the G2/M checkpoint in cooperation with GADD45α and GADD45γ after the cells were exposed to ultraviolet radiation. Inowa et al. [26] studied the stem cell-enriched side population (SP) cells in tumors. The inhibition of GADD45B significantly reduced the viability and invasiveness of necB SP cells, suggesting that the high expression of GADD45B promoted the proliferation and migration of SP cells, which might determine their stem cell-like phenotype and have an important impact on their drug resistance. In addition, Cheng et al. [27] reported that GADD45B could induce the downregulation of cyclin B1 and the disassociation of CDC2/cyclin-B1, thus inhibited cell proliferation and induced mitosis delay in cancer cells. GADD45B inhibited the activity and proliferation of HONE1-R cells in the normal physiological state [28]; our present study showed that the overexpressed GADD45B could effectively increase the activity and proliferation of HONE1-R cells and enhanced their resistance to radiation therapy after radiation exposure.

5 Conclusion

In conclusion, a stable and genetically resistant HONE1-R cell line was constructed. The transcriptome sequencing analysis showed that the MAPK signaling pathway was the most important signaling pathway of radiation resistance in HONE1-R cells. More attention should be paid to the key gene GADD45B. This gene can inhibit the viability and proliferation of HONE1-R cells under normal physiological conditions, but it can effectively enhance the viability and proliferation of HONE1-R cells under radiation stimulation and increase the radioresistance of HONE1-R cells. Therefore, GADD45B may become a new target for radiotherapy.

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