 Slug inhibition increases radiosensitivity of nasopharyngeal carcinoma cell line C666-1

HONGXIA YANG1*, GANG ZHANG2*, XIAOLIN CHE3 and SHUDONG YU4

1Department of Otorhinolaryngology, Maternal and Child Health Hospital of Tai'an; 2Department of Otolaryngology, Affiliated Hospital of Taishan Medical University, Tai'an, Shandong 271000; 3Department of Otolaryngology, Shandong University of Traditional Chinese Medicine, Jinan, Shandong 250011; 4Department of Otolaryngology, Qianfoshan Hospital Affiliated to Shandong University, Jinan, Shandong 250014, P.R. China

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Abstract. Slug is associated with the radioresistance of nasopharyngeal carcinoma (NPC) and the main current approach of treatment for NPC is radiotherapy. Hence, the aim of the current study was to determine the effect of Slug silencing on the radiosensitivity of NPC cells. Lentiviral-mediated transfection of Slug RNA interference (RNAi) in NPC cell line C666-1 was performed in vitro. Following Slug inhibition, its expression was detected using western blotting. A clonogenic survival assay and flow cytometry were then performed to evaluate the clonogenic cell survival, cell cycle distribution and apoptosis of C666-1 cells following irradiation. The results indicated that Slug RNAi decreased cell proliferation, and increased cell apoptosis and G0/G1 arrest. Thus, lentiviral-mediated transfection of Slug RNAi enhanced the radiosensitivity of the NPC cell line C666-1, and Slug may therefore be a potential target to improve radiotherapy in treatment of NPC and reduce the radioresistance of NPC.

Introduction

Nasopharyngeal carcinoma (NPC) is endemic in Southern China (1). It was the eighth most common type of cancer in 2010, and accounted for 3.5% of all new cancer cases worldwide (2). Genetic susceptibility and Epstein-Barr virus infection are important etiological factors in NPC (3). For decades, the primary treatment strategies used for NPC were chemotherapy and radiotherapy (4,5). Recently, the platinum doublet of fluorouracil plus cisplatin reigned as a first-line treatment strategy for chemotherapy (2). However, its efficacy remains controversial. Radiotherapy was the mainstay treatment for NPC due to its relatively high radiosensitivity and deep-seated anatomical position, which made surgical resection challenging (6). In addition, great technological advances have been made in radiotherapy, including three-dimensional conformal radiotherapy and intensity-modulated radiotherapy (7,8). However, due to irradiation resistance, some patients with NPC present with metastases following radiotherapy (5). Hence, enhancing the radiosensitivity of NPC may provide a novel treatment strategy for NPC.

Slug is a conserved zinc finger transcription factor, which belongs to the Snail family and presents an anti-apoptotic effect by regulating the transactivation of p53 upregulated modulator of apoptosis (PUMA) and the expression of B cell lymphoma-2 (Bcl-2) and Bcl-2-associated X protein (9,10). It has been demonstrated that Slug-deficient cells are radiosensitive to DNA damage (11,12). Slug inhibition also increases the radiosensitivity of HSC3 and HSC6 cells by upregulating PUMA in oral squamous carcinoma (13). In addition, Slug expression inhibits calcitriol-mediated sensitivity to radiation in colorectal cancer (14) and upregulates radiation-induced PUMA in cholangiocarcinoma (15). However, whether Slug inhibition may enhance radiosensitivity of NPC remains unknown.

Radio-gene therapy has been developed as a novel strategy, which combines traditional radiotherapy with gene therapy (16,17). Due to radiation resistance, radiotherapy is sometimes ineffective, resulting in worse side effects (18). Therefore, it is necessary to understand the radiosensitive tumor targets and mechanisms underlying the development of radioresistance.

Thus, the aim of the present study was to investigate whether Slug inhibition may increase the radiosensitivity of NPC cell line C666-1. Following combined treatment of lentivirus-mediated Slug RNA interference (RNAi) transfection with X-ray irradiation, the expression of Slug was decreased, which resulted in increased irradiation (IR)-induced G0/G1 arrest and cell apoptosis of C666-1. These findings may offer novel insights into radiotherapy with gene therapy in the treatment of NPC.

Correspondence to: Dr Shudong Yu, Department of Otolaryngology, Qianfoshan Hospital Affiliated to Shandong University, 16766 Jingshi Road, Jinan, Shandong 250014, P.R. China

E-mail: articlesreview@163.com

*Contributed equally

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Materials and methods

Cell culture. Human NPC cell line C666-1 was obtained from the Xiangya Central Experiment Laboratory (Central South University, Hunan, China). Cells were cultured in RPMI-1640 media (GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., Hangzhou, China), 50 U/ml penicillin G and 50 U/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 5% CO₂ in a humidified atmosphere at 37°C. The cells were passaged every 3 days.

Irradiation procedure. Irradiation was performed with a single dose of X-rays ranging from 1-8 Gy using a linear accelerator ( Trilogy, Austin, TX, USA) with 6 MV photons/100 cm focus-surface distance at room temperature. The dose rate was 4.0 Gy/min, which was determined by Fricke's chemical dosimeter (19).

Construction of lentiviral vectors. In order to produce lentivirus expressing RNAi specific for the Slug gene, the RNAi sequence for human Slug (GGAAATATGTTAGCGCTGGGC GCC) was identified using the BLOCK-it RNAi Designer program (Invitrogen; Thermo Fisher Scientific, Inc.) and the negative control construct (control RNAi) was created using a scrambled sequence (GAA  CCG TGT CTT CCT CAG TAT C). A pair of primers (AgeI and EcoRII) were annealed and cloned into the pGCSIL-Slug-shRNA-LV vector ( Shanghai Genechem Co., Ltd., Shanghai, China). Following confirmation of the constructed plasmids by DNA sequencing, lentiviral vector DNA and packaging vectors (Invitrogen; Thermo Fisher Scientific, Inc.) were then transfected into 293T cell (Sangon Biotech, Co., Ltd., Shanghai, China) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Following construction of the plasmids by DNA sequencing, lentivector DNA and packaging vectors (1 µg/ml; pHelper 1.0 and pHelper 2.0; Shanghai Genechem Co., Ltd.) were then transfected into 293T cells (Sangon Biotech, Co., Ltd., Shanghai, China) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Following 48 h of culture, supernatants containing lentiviruses, including pGCSIL-Slug-shRNA-LV and pGCSIL-neg-shRNA-LV were harvested, respectively. Purification was then performed at 1,000 x g and 4°C for 2 min using ultracentrifugation (Himac CT15RE; Hitachi, Tokyo, Japan) and the titer of lentivirus was determined. The lentiviruses were stored for 1 week in -80°C and prepared for infection.

Infection of lentivirus. C666-1 cells were cultured in 60-mm dishes with 5x10⁵ cells in each plate. Cells were cultured in complete medium with lentiviruses at a multiplicity of infection of 10 for 24 h at 37°C. Fresh culture medium was then used to substitute the old media. Total protein was isolated and the expression of Slug was detected by western blot analysis.

Clonogenic cell survival. Cells (5x10⁴) were seeded in culture dishes with Dulbecco's modified Eagle's medium (10% fetal bovine serum; Thermo Fisher Scientific, Inc.) and irradiated the next day at the 4 Gy dose according to the reference (20).

Cell cycle analysis. A total of 24 h following irradiation, cells were trypsinized, plated in 60-mm dishes and incubated for 14 days at 37°C to allow colony growth. Colonies were fixed with 10% methanol and 10% glacial acetic acid for 5 min. The colonies were washed twice with PBS and harvested. The colonies were then centrifuged at 1000 x g and 4°C for 2 min. Cell viability was calculated using (number of colonies counted)/[(number of colonies plated) x (plating efficiency)]. The plating efficiency was calculated as: (number of colonies counted)/(number of cells plated). The relative proportion of cells in each cell cycle phase was determined using a flow cytometer (FACSCalibur™; BD Biosciences, Franklin Lakes, NJ, USA). The relative proportion of cells in the individual cell cycle phase was determined by the flow cytometry data using FCSExpress 3.0 (De Novo Software, Glendale, CA, USA).

Apoptosis assay. A total of 24 h following exposure to 4 Gy X-rays, cells were stained with Annexin V-alphycocyanin (APC)/PI (Abcam) for 15 min at room temperature to measure cell apoptosis. A total of 1.0x10⁵ cells were washed twice with ice-cold PBS and incubated for 30 min in binding buffer.
at room temperature. Fluorescence-activated cell sorting analysis for Annexin V-APC/PI staining was performed by flow cytometry (FACSCalibur™; BD Biosciences) with FCS Express 3.0 software. Cells that stained positive for Annexin V and negative for PI were undergoing apoptosis. Cells that stained positive for Annexin V and PI were either in the end of stage of apoptosis, undergoing necrosis or were already dead. Cells that stained negative for Annexin V and PI were alive and not undergoing measurable apoptosis.

**Statistical analysis.** All experiments were performed in triplicate and data were expressed as the mean ± standard deviation. Statistically significant differences between groups were determined using one way analysis of variance followed by the Tukey's post hoc multiple comparison test using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference. The survival curve was drawn using Sigma Plot 12.0 (Systat Software, Inc., San Jose, CA, USA).

**Results**

**Slug is downregulated by lentivirus-mediated RNAi in C666-1 cells.** A lentivirus vector system derived from HIV-1 was used to express short hairpin RNA (shRNA) directed against Slug to downregulate its expression in C666-1 cells. The effect of lentivirus-mediated RNAi knockdown of Slug on its expression was determined using western blot analysis. The expression of Slug in the KD group was significantly decreased compared with that in the CON and NC groups (Fig. 1). The difference in Slug expression between the CON group and the NC group was not significant. Thus, these results confirmed the downregulation of Slug in the C666-1-transfected cell line.

**Slug inhibition increases radiosensitivity and decreases cell survival of C666-1.** The effects of Slug inhibition in combination with X-ray irradiation on C666-1 cell survival was investigated using a clonogenic survival assay. The results indicated that Slug inhibition significantly increased cell sensitivity to X-ray irradiation compared with the level of sensitivity observed in the NC group and CON group (Fig. 2). The values of SF2, D0, Dq and N for the KD group were all decreased significantly compared with those in the CON and NC groups (Table I). SF2 was reduced to 57.6% in the KD group from 85.5% in the CON group, and the sensitization enhancement ratio was 1.48 (data not shown). In addition, the results indicated that cell colony forming efficiency was decreased in the KD group compared with that observed in the NC group following irradiation treatment (Fig. 2). There were few visible colonies that were able to be easily seen with the naked eye that were irradiated with 4 Gy in the KD group in comparison with the CON and NC groups (Fig. 2).

**Effect of Slug inhibition combined with X-ray irradiation on the C666-1 cell cycle.** The impact of Slug inhibition and X-ray irradiation on the cell cycle was also investigated. Analysis indicated that Slug inhibition and X-ray irradiation induced a significant increase in the G0/G1 phase (56.09±1.07% NC group vs. 72.21±1.38% KD group) and decrease in the S phase in the proportion of cells (32.13±1.14% NC group vs. 15.17±1.16% KD group) in the KD group compared with the proportion in

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Table I. Survival curve parameters fitting the data into a multi-target single-hit model.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CON</th>
<th>NC</th>
<th>KD</th>
</tr>
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<tbody>
<tr>
<td>D0</td>
<td>2.269</td>
<td>2.226</td>
<td>1.988&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dq</td>
<td>2.128</td>
<td>2.078</td>
<td>1.338&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>N</td>
<td>3.724</td>
<td>3.596</td>
<td>1.916&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>SF2</td>
<td>0.855</td>
<td>0.823</td>
<td>0.576&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

D0, mean lethal dose; Dq, quasi-threshold dose; N, extrapolation number; SF2, surviving fraction at 2 Gy; CON, control group consisting of untreated C666-1 cells; NC, negative control consisting of C666-1 cells infected with pGCSIL-neg-shRNA-LV; KD, Slug RNA interference group consisting of C666-1 cells infected with pGCSIL-Slug-shRNA-LV. *P<0.05 vs. D0 in the NC and CON groups; †P<0.05 vs. Dq in the NC and CON groups; ‡P<0.05 vs. N in the NC and CON groups; §P<0.05 vs. SF2 in the NC and CON groups.
the NC group (Fig. 3). The frequency of cells with a down-regulated expression of Slug in the S phase was significantly decreased in the KD group compared with that in the CON group and NC group. These results indicate that Slug inhibition combined with X-ray irradiation increased the number of cells in the $G_0/G_1$ phase of the cell cycle.

**Discussion**

NPC is an epithelial malignancy arising in the head and neck region of the body (22). It is prevalent in Southern China with an annual incidence of 20/100,000 people (23). The ‘gold standard’ of treatment for NPC is radiotherapy, which has a cure rate of >90% in patients with early-stage NPC (24). However, due
to radiation resistance, the efficacy of radiotherapy is limited, especially in advanced stages of NPC. Hence, decreasing the radiation resistance and increasing radiosensitivity is a method that may improve the efficacy of radiotherapy in patients with NPC.

The Slug protein belongs to the Snail superfamily of zinc finger transcription factors, which is associated with embryonic development, regulation of carcinogenesis in various cancer types and anti-apoptosis (25-29). It has been demonstrated that Slug may be involved in the radiosensitivity of different types of cancer, including colorectal cancer, cholangiocarcinoma and ovarian cancer (14,15,30,31). However, there have been few studies on the function of Slug in association with radioresistance in NPC. Therefore, the present study investigated the effect of Slug inhibition on the radiosensitivity of NPC cell line C666-1.

The effect of Slug inhibition on the radiosensitivity of NPC cell line C666-1 was investigated by downregulating the expression of Slug by infecting the cells with Slug-specific RNAi-expressing lentivirus. Following downregulation, the expression of Slug was significantly decreased. A clonogenic survival assay was then performed, which suggested that the downregulation of Slug decreased clonogenic survival. Furthermore, a cell apoptosis assay was applied to measure cell apoptosis following X-ray irradiation in combination with Slug downregulation. The results indicated that Slug inhibition induced cell apoptosis following irradiation. Slug inhibition may therefore, increase the radiosensitivity of NPC via the induction of apoptosis and cell cycle arrest.

It has been demonstrated that Slug serves a role in the radioresistance of several types of cancer (31,32). However, the mechanisms underlying the radioresistance of cancer remain unclear. PUMA is a pivotal protein in apoptosis and it has been suggested that PUMA may increase sensitivity to radiation-induced apoptosis in different types of cancer (15,33,34). Slug is a suppressor of PUMA transcription, which inhibits the expression of PUMA in cell survival (35). Therefore, it may be hypothesized that Slug, as a radioprotection agent, may serve an important role in decreasing radiosensitivity by inhibiting the expression of PUMA. Following the downregulation of Slug, it has been demonstrated that PUMA increases cell apoptosis and sensitivity to radiation (13). In future studies, further investigation of the pathways involved in the development of NPC is required.

In conclusion, the results of the current study indicated that Slug may be a potential target of radio-genie therapy to increase the radiosensitivity of NPC.

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