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The clinical relationship between the slug-mediated Puma/p53 signaling pathway and radiotherapy resistance in nasopharyngeal carcinoma

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Abstract. – OBJECTIVE: To explore the clinical relationship between the Slug-mediated Puma/p53 signaling pathway and radiotherapy resistance in nasopharyngeal carcinoma.

PATIENTS AND METHODS: Forty surgical specimens were collected from nasopharyngeal carcinoma patients treated at our hospital between February 2010 and February 2015. Twenty patients with poorly differentiated nasopharyngeal carcinoma with and without radiotherapy resistance were included in the experimental and control groups, respectively. Slug, Puma, and p53 expression were quantified in all tissues using fluorescence quantitative polymerase chain reaction, enzyme-linked immunosorbent assay (ELISA), Western blotting, and immunohistochemistry.

RESULTS: Slug and p53 mRNA levels were significantly higher in the experimental group than in the control group ($p < 0.01$). Puma mRNA levels were significantly lower in the experimental group than in the control group ($p < 0.01$). Slug protein expression was significantly higher in the experimental group (6.07 ± 0.203 μg/L) than in the control group (1.24 ± 0.171 μg/L) ($p < 0.01$). p53 protein expression was significantly higher in the experimental group (4.28 ± 0.108 μg/L) than in the control group (0.63 ± 0.101 μg/L) ($p < 0.01$). Puma protein expression was significantly lower in the experimental group (0.43 ± 0.11 μg/L) than in the control group (3.37 ± 0.112 μg/L) (v < 0.01). The number of Slug, Puma, and p53-positive cells in the experimental group and the control group were quantified; these values confirmed the ELISA and Western blot findings.

CONCLUSIONS: Slug downregulated the Puma protein expression signaling pathway and promoted radiotherapy resistance in poorly differentiated squamous cell carcinoma tissue, in a p53-independent manner.

Key Words:
Slug, Puma, p53, Nasopharyngeal carcinoma, Radiotherapy resistance.

Introduction

Nasopharyngeal carcinoma (NPC) is a malignant head and neck tumor that mainly affects adults aged 40-50 years[1-3]. There are approximately 1 million NPC patients in China, and NPC prevalence is reported to be increasing by 0.32% per year[4]. NPC has a significant impact on patients, their families, and the society. Recent advances in medical technology have improved our understanding of NPC. Because the primary site for NPC remains difficult to detect and symptoms have a late onset, NPC is often undiagnosed or misdiagnosed. Thus, NPC is usually detected when the disease is already in advanced stages[5]. Although the current preferred treatment for NPC is radiotherapy and/or chemotherapy, such treatments are not curative, and many patients develop resistance. Therefore, the development of new treatments for NPC has become an important focus[6].

Slug is a zinc finger protein belonging to the Snail protein family, and it plays an important role in neurulation and normal development of the nerve trunks in humans[7,8]. Slug overexpression in gastric cancer tissue promotes tumor metastasis/infiltration and leads to the generation and deterioration of malignant mesenchymal cells in mice[9].

Slug expression has been associated with human cancers and is related to cancer morbidity[10]. However, the correlation between Slug expression and radiotherapy resistance in NPC has not been investigated. In this study, we conducted a preliminary analysis of the correlation between Slug expression and radiotherapy resistance in NPC, with an additional focus on related signal transduction pathways. Our aim was to provide a theoretical and experimental basis for future research into radiotherapy resistance in NPC.

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Patients and Methods

We used 40 surgical specimens obtained from NPC patients treated by radiotherapy at our hospital between February 2010 to February 2015. Twenty samples were from NPC patients with radiotherapy resistance (experimental group). The average age in the experimental group was 47.2 ± 7.8 years (male *vs.* female, 11 *vs.* 9). Twenty samples were from NPC patients with radiotherapy sensitivity (control group). The average age in the control group was 50.63 ± 10.6 years, and the male-to-female ratio was 10:10. The metastatic status of patients lymph nodes was determined before the operation; no lymphatic metastasis was detected in any of the patients. Approval for the study was obtained from the Ethics Committee of the Second People's Hospital of Wuxi.

Fluorescent Quantitative PCR

RNA was extracted from frozen tissue using an RNA Plus kit, according to the manufacturer's instructions. PCR was performed using the TAKARA fluorescent quantitative PCR system. Primers were synthesized by Shanghai Biological Engineering Technology Co., Ltd. The primer sequences are shown in Table I. Reagents for fluorescent quantitative PCR and consumable items were purchased from TAKARA Company (Dalian, Liaoning, China).

Enzyme-linked Immunosorbent Assay (ELISA)

Slug, p53 up-regulated modulator of apoptosis (Puma), and p53 protein expressions were quantified in tissue samples using ELISA kits (Axygen, Silicon Valley, CA, USA), according to the manufacturer's instructions. Standard protein specimens were used to generate the standard curve. Slug, Puma, and p53 expressions in each sample were calculated according to the standard curve.

Western Blotting

Frozen tissue was initially ground and mixed with 300 μL of protein extraction solution containing 10 μL of protease inhibitors, before being centrifuged for 10 min at 12000 r/min. The supernatant was collected for analysis. To detect protein expression in the tissues, 10 μL of supernatant and loading buffer were loaded onto SDS gels and separated by electrophoresis. Proteins were transferred onto transfer membranes and incubated in anti-Slug, anti-Puma, and anti-p53 primary antibodies (1:250) overnight at 4°C. Membranes were incubated with the second antibody conjugated to horseradish peroxidase (1:1250) for 1 h. Membranes were stained with diaminobenzidine and membrane bands detected using the Fluorchem 9900 imaging system (BC, Tokyo, Japan). Protein expression was quantified by measuring the integral optical density of each protein band. The following primary antibodies were used for Western blotting: mouse anti-human Slug monoclonal antibody, mouse anti-human Puma primary antibody, and mouse anti-human p53 primary antibody (ABM Company, Silicon Valley, CA, USA). The chemiluminescence reagent was purchased from Thermo Corporation (Waltham, MA, USA). The fluorescent quantitative PCR instrument was purchased from ABI Step Plus and Western blotting apparatus was purchased from Bio-Rad (Hercules, CA, USA).

Table I. Primer sequences.

Gene	Sequence
Slug-F	ATGCGTCGATGCTACGTAGCATAC
Slug-R	CTGATGCTGGTCGATCAGTCCGTAC
GAPDH-F	GTCGATGGCTAGTCGTAGCATCGAT
GAPDH-R	TGCTAGCTGGCATGCCCGATCGATC
PUMA-F	CGTCGTAGCTGATCGATCGATCACG
PUMA-R	CGTGCTGCTGATGCTAGCTAATCGTC

Immunohistochemistry

Immunohistochemistry was performed as previously described[5]. Positive staining was quantified by the KI index (Keep identity). The KI index refers to the number of positive cells in each field of view.

Statistical Analysis

Statistical analysis was performed using SPSS 20.0 software (IBM, City of New York, NY, USA). Data were expressed as mean ± standard deviation. The single factor analysis method was used to analyze the differences between groups. p-values of < 0.05 indicated a statistically significant difference.

Results

Expression of Slug, Puma, and p53 mRNA

We quantified Slug, Puma, and p53 mRNA expressions by fluorescent quantitative PCR (Figure 1). Slug and p53 mRNA expressions were significantly higher in the experimental group than in the control group ($p < 0.001$). Pu-

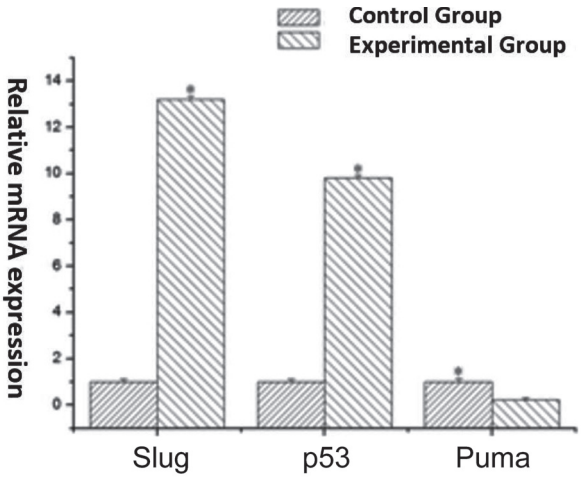

Figure 1. The relative expressions of Slug, PUMA, and p53 mRNA between groups. *:Statistically significant difference.

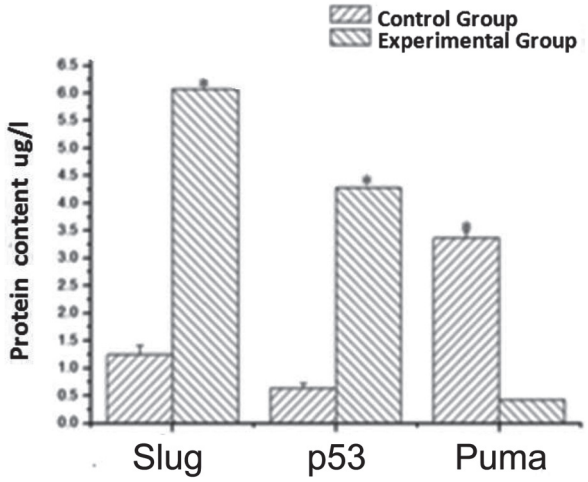

Figure 2. Measurement of Slug, PUMA, and p53 protein expressions by ELISA.

ma mRNA expression was significantly lower in the experimental group than in the control group ($p < 0.05$).

Slug, Puma, and p53 Protein Expressions Measured by ELISA

Slug, Puma, and p53 protein expressions were measured using ELISA kits (Figure 2). Slug and p53 protein expressions were significantly higher in the experimental group than in the control group ($p < 0.01$) (Table II). Puma expression was significantly lower in the experimental group than in the control group ($p < 0.01$). This result was consistent with the PCR results.

Measurement of Slug, Puma, and p53 Protein Expressions by Western Blotting

Slug, Puma, and p53 protein expressions in patient tissues were determined by Western blotting (Figure 3). Slug and p53 expressions were significantly higher in the experimental group than in the control group. Puma expression was significantly lower in the experimental tissues than in the control tissues. These findings were consistent with the ELISA results.

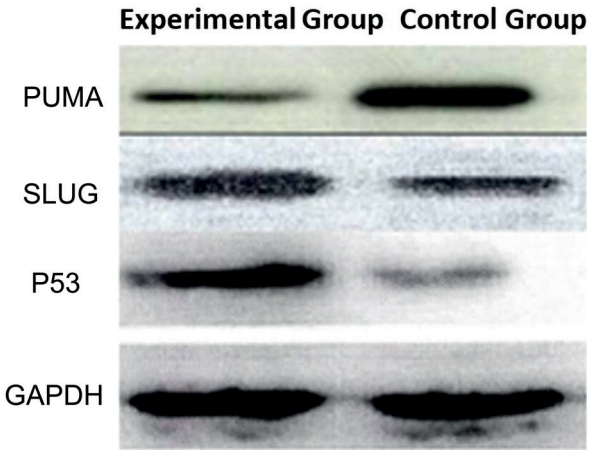

Figure 3. Quantification of Slug, PUMA, and p53 protein expressions by Western blotting.

Evaluation of Slug, Puma, and p53 protein expressions by immunohistochemistry

We investigated Slug, Puma, and p53 protein expressions in tissue sections by immunohistochemistry (Figure 4). Slug expression was mainly localized in the cytoplasm and nucleus. p53 staining was

Table II. Measurement of Slug, PUMA and p53 protein expressions by ELISA.

Group	Case load	Slug (μg/L)	p53 (μg/L)	PUMA (μg/L)
Control group	20	1.24 ± 0.171	0.63 ± 0.101	3.37 ± 0.112
Experimental group	20	6.07 ± 0.203	4.28 ± 0.108	0.43 ± 0.11
p	> 0.05	< 0.05	< 0.05	< 0.05

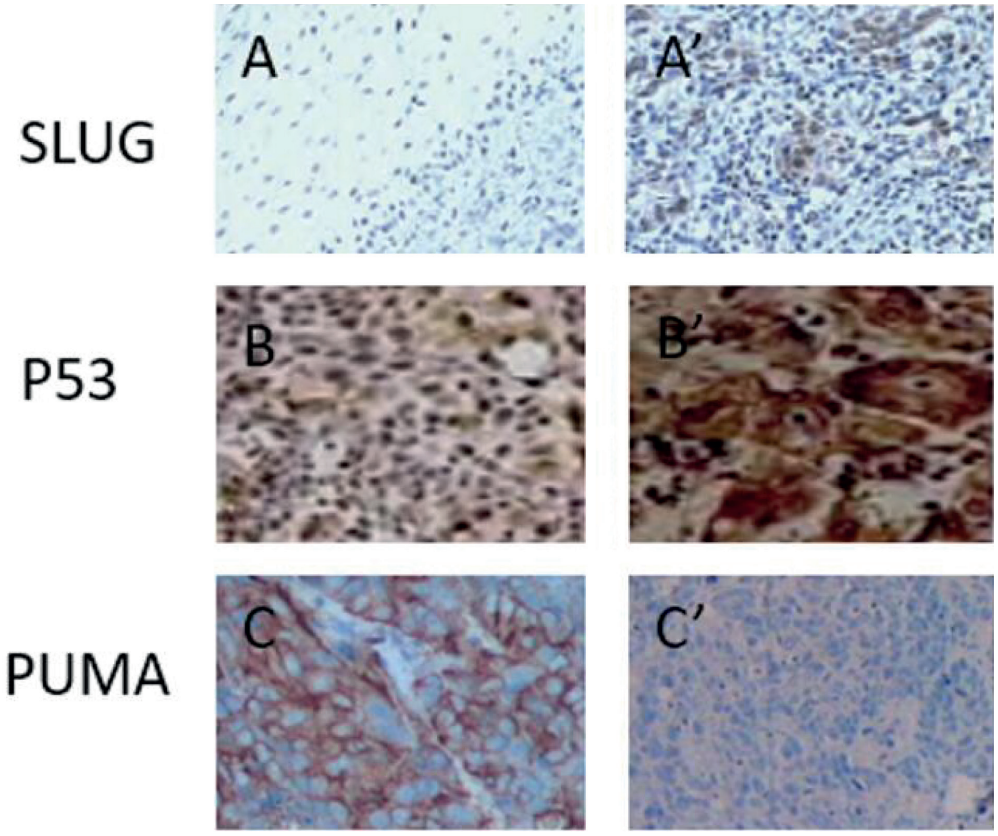

Figure 4. Immunohistochemistry of Slug, PUMA, and p53 in ***(A)*** control tissues and ***(B)*** experimental tissues.

mainly distributed in the cytoplasm, and Puma staining was mainly observed in the cell membrane. The number of Slug-positive and p53-positive cells were significantly higher in experimental tissues than in the control tissues (Table III).

Discussion

NPC tumors are located in the nasopharyngeal mucosa, which is frequently involved in infectious processes. NPC pathogenesis is poorly understood; therefore, current treatments are ineffective[11,12]. MicroRNAs regulate signal transduction pathways by RNA polymerase II during NPC progression[13,14], including tissue transferase and polymerase miRNA activity[15]. These pathways regulate the proliferation and migration[16] of cancer cells through interactions with AGO protein. The combined action of Slug and Twist can inhibit tumor occurrence, development, and invasion[17,18]. Furthermore, Slug overexpression has been closely correlated with the development of cancers, such as gastric, lung, breast,

Table III. Quantification of Slug-, PUMA-, and p53-positive cells.

Group	Case number	Cell population	Slug		p53		PUMA	
			Positive	Negative	Positive	Negative	Positive	Negative
Control group	20	400	26	374	39	361	372	28
Experimental group	20	400	327	63	308	92	21	379
p		< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	

and ovarian cancers[19] Slug protein is reported to be upregulated in many cancers, and abnormal Slug expression can inhibit the proliferation and migration of pancreatic cancer cells[20]. Although Slug expression has been correlated with many cancers[21], the role of Slug in NPC has not been investigated.

Puma is positively regulated by p53 and participates in apoptosis[22]. Puma is weakly expressed in the healthy tissue, but it increases rapidly in response to external stimulation[23-24]. Also, Puma can induce Bax-mediated mitochondrial apoptotic pathways by interacting with p53 and members of the Bcl-2 family[25,26].

We explored the correlation between Slug, Puma, and p53 expressions and radiotherapy resistance in NPC. Slug and p53 expressions were high in radiotherapy-resistant tissues, whereas Puma expression was high in radiotherapy-sensitive tissues. This indicated a positive correlation between Slug and p53 expressions and radiotherapy resistance in NPC. It also showed that Puma expression correlates negatively with radiotherapy resistance in NPC. Furthermore, we showed that p53 and Puma expression were negatively correlated. These findings are contradictory to previous findings, and it may be because of differences in pathogenesis between tumor types of NPC[26]. Moreover, the signaling pathways involved in different cancers and involved proteins are different, and the same gene can have different effects in different tumors.

Immunohistochemistry revealed that there were some Slug-positive cells in radiotherapy-sensitive and radiotherapy-resistant tissues. NPC pathogenesis likely involves many genes and different individual constitutions and different courses of the disease may cause differences in the expression of Slug-related signaling components between tumors.

Conclusions

Our findings showed that Slug enhances radiotherapy resistance in NPC and promotes tumor progression through the p53 signaling pathway. On the other hand, Puma can decrease the resistance of NPC to radiotherapy through the p53 signaling pathway. However, this regulation was dose-dependent. Therefore, Slug may be involved in different signaling pathways at different stages of NPC; this aspect should be investigated further in the future.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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