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REGENERATING GENE IA PREDICTS RADIOSENSITIVITY AND SURVIVAL IN NASOPHARYNGEAL CARCINOMA

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Nasopharyngeal carcinoma (NPC) is a common malignancy in Southern China and Southeast Asia. Radiotherapy is the main treatment option. However, radiotherapy does not benefit all patients because there is no known precise biomarker that can be used for screening radioresistant patients. Genetic predisposition is closely related to tumor development, therapeutic response, and prognosis. The relationship between regenerating gene IA (REGIA) and NPC is unclear. This study aimed to retrospectively analyze the association between REGIA expression and metastasis, radiosensitivity, and survival in patients with NPC as well as assess the effect of radiation on REGIA expression *in vitro*. Immunohistochemical staining was used to detect REGIA. The relationship between REGIA expression in radioresistant NPC and the prognosis of CNE1 NPC cells were analyzed using quantitative real-time polymerase chain reaction and Western blotting. We found that increased doses of radiation in CNE1 cells significantly decreased REGIA expression ($P<0.05$). The overall rate of REGIA-positive expression was 47.15% in NPC tissues and 45.00% and 61.02% in radiosensitive and radioresistant cases, respectively, showing significant differences ($P<0.05$). A REGIA-positive protein expression rate had a negative correlation with radiosensitivity in NPC ($r= –0.109$, $P=0.047$). Both REGIA-positive and REGIA-negative expression strongly predicted the overall survival rate and progression-free survival of NPC patients ($P<0.01$). A multivariate analysis indicated that REGIA was an inverse prognostic factor in NPC patients (REGIA-positive expression: hazard ratio (HR)=2.139, 95% confidence interval (CI)=1.56–2.94, $P<0.001$ and REGIA-negative expression: HR=1.958, 95% CI=1.42–2.69, $P<0.001$). In conclusion: Radiation can affect REGIA expression. The REGIA expression level correlated with radioresistance and a poor prognosis. In addition, REGIA expression might act as a potential therapeutic target and prognostic predictor in NPC patients.

Key words: *regenerating gene IA, nasopharyngeal carcinoma, radiosensitivity, radioresistance, prognosis, cell irradiation, chemotherapy, survival, biomarkers*

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a rare malignancy in most parts of the world, with an incidence in humans of well under 1 in 100,000 per year (1). However, there is a much higher incidence of NPC in China. Radical radiotherapy is the standard therapy option; however, this treatment often fails due to radioresistance, resulting in residual and recurrent lesions (2). Improved radiosensitivity, leading to a reduction in residual and recurrent lesions, would improve the prognosis for patients diagnosed with NPC (3, 4). Hence, it is desirable to find the biomolecules that can identify the sensitivity of NPC cells to radiotherapy.

Regenerating gene IA (REGIA), a subtype of the human REG I gene, is located on chromosome 2p12 and contains 166 amino acids (5). Regenerating gene IA occurs in gastroenterological cancers (6-10) and autoimmune disease (11, 12) and is also a factor in lung (13), breast (14), and bladder (15) carcinogenesis. Overexpression of REGIA enhances cell invasion and viability and suppresses the apoptosis of human gastric and bladder cancer cells. This occurs through the activation of phosphatidylinositol 3-kinase/protein kinase B (Akt)-glycogen synthase kinase 3 beta (PI3K/Akt-GSK3β) signaling, the suppression of the phosphorylation of Akt or GSK3β signaling (16-19), the activation of extracellular signal-regulated kinase (ERK) (20), and the stimulation of B-cell lymphoma-extra-large (Bcl-xL) expression (21, 22).

Of the factors mediating the REGIA functions, its receptor, exostosin tumor-like 3, regulates REGIA's effects on neurite outgrowths (23). In salivary ductal cells, interleukin-6 (IL-6) stimulation induces REGIA transcription through signal transduction and activation of transcription 3 (STAT3), which in turn binds to the REGIA promoter because STAT3 contains the consensus sequence of STAT binding (11) and a Janus kinase/STAT pathway under inflammatory conditions (24). Regenerating gene IA has varied biological mechanisms that regulate different cell functions; in pancreatic-derived cells, high intracellular levels of REGIA lead to decreased cell growth and induced apoptosis *via* inactivation of mitogen-activated protein kinase phosphatases-1 (25).

However, REGIA is susceptible to anticancer treatments. Hayashi *et al*. reported that REGIA was overexpressed in squamous cell esophageal carcinoma prior to chemoradiotherapy (7, 26). Wakita *et al*. found that REGIA increased both chemosensitivity and radiosensitivity in squamous esophageal cancer cells. They transfected TE-5 and TE-9 squamous esophageal cancer cells with REGIA and activated the expression of Jun proto-oncogene (c-Jun) mRNA *via* the phospho-c-Jun protein mediated *via* the c-Jun N-terminal kinase (JNK) and ERK pathways (27, 28). In addition, REGIA expression made gastric cancer cells resistance to S-1 and cisplatin treatment (29). Sato *et al*. reported that promoter region CpG methylation enhanced REGIA expression and regulated melanoma lines susceptible to dacarbazine and cisplatin (30), suggesting that REGIA expression status might be used to assess both chemosensitivity and radiosensitivity. Evidence has also indicated that REGIA expression could monitor the therapeutic response and/or predict the patient's prognosis (31-34) in head and neck cancers (35, 36), although the underlying mechanism is unclear, and the results were inconsistent.

In light of these previous findings, this study speculated that REGIA's status as a molecular predictor might increase the benefits of radiotherapy in NPC patients, thus increasing their survival.

MATERIAL AND METHODS

Patients and clinical characteristics

Study patients had diagnoses that met the following eligibility criteria: 1) non-keratinizing or undifferentiated NPC (World Health Organization (WHO) type II or type III cancer) (37); 2) stage I to stage IV (A–B) according to the International Union Against Cancer tumor, node, metastasis (TNM) classification (2002) (38); 3) an untreated primary tumor; 4) the absence of serious liver and renal dysfunction; 5) no distant metastases at the time of initial diagnosis; and 6) the availability of medical records and sufficient pretreatment tumor biopsy specimens. A total of 439 patients with biopsy-proven NPC, diagnosed from June 2009 to December 2017, were enrolled in this study. The T stage criteria are as follows: T1: the maximum diameter of the primary lesion is less than 2 cm; T2: the maximum diameter of the primary lesion is larger than 2 cm and less than 4 cm; T3: the maximum diameter of the primary lesion is larger than 4 cm and less than 6 cm; T4: the maximum diameter of the primary lesion is larger than 6 cm or other criteria. The clinicopathological characteristics of all included patients are listed in *Table 1*.

All patients signed an informed consent form before the donation of their biopsies, and approvals were acquired from the Institute Research Ethics Committee of the Affiliated Hospital, Hainan Medical College, before these clinical materials were used for research (HNM-2016LL-056). All methods were performed in accordance with the Declaration of Helsinki. Clinical follow-up data were obtained through the patients' medical records.

Patient treatment

The enrolled patients received radical irradiation alone through intensity-modulated radiotherapy. The radiation protocol was a total dose of 70–78 Gy to the primary tumor (average dose of 71.8 Gy) and 48–74 Gy to the positive cervical lymph nodes. All patients were treated with one fraction daily for five days each week, with 2 Gy per fraction.

Follow-up and radiosensitivity assessment

All patients were evaluated weekly during the treatment period. After the completion of radiotherapy, the patients were followed up with monthly for the first three months, every three months through three years, every six months for the next two years, and then annually. Each follow-up evaluation included a physical examination, a flexible endoscopy, basic serum chemistry, a chest x-ray, and liver and abdomen ultrasounds. A magnetic resonance imaging examination of the head and neck was performed approximately three months after the completion of radiotherapy and every 6–12 months thereafter.

At three months after the completion of radiotherapy, the treatment efficacy was assessed based on the Response Evaluation Criteria in Solid Tumors (version 1.1), and the following evaluation responses were given (39): complete response (CR), partial response, stable disease, progressive disease, or no assessment. An evaluation of CR was regarded as radiosensitive, and the others were regarded as radioresistant.

Cell culture

The human NPC cell line CNE1 (Ming Shang Shan Bio, China [purchased from NECB, 2018.05]), a genetically stable nasopharyngeal carcinoma cell line derived from tumor cells of patients with locally advanced nasopharyngeal carcinoma without lymph node metastasis after primary culture, was used in this study. It was cultured in RPMI-1640 medium (Hyclone, Logan, Utah, USA. Cat. No. SH30809.01B) and supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah, USA. Cat. No. SH30087.01), 100 units of penicillin/ml, and 100 μg of streptomycin/ml (Hyclone, Logan, Utah, USA. Cat. No. SH30010) in a humidified chamber at 37°C with 5% CO_2.

Cell irradiation

The cells were trypsinized and seeded into 6-well plates at a density of 10^4 cells and then treated with 0, 0.5, 2, 4, and 10 Gy of irradiation, respectively. The experimental doses were calculated as multiples of radiotherapy fraction, which the conventional segmentation dose is 1.8Gy-2.0Gy. After the cells became adherent, they were irradiated at defined doses using a Rad Source R2000 x-ray irradiator (1.0 Gy/min, 160 kV, 25 mA, 0.3 mm copper filters; Rad Source Tech, Suwanee, Georgia, United States). After 48 h of incubation, the cultures were fixed and stained with Giemsa stain.

RNA isolation and quantitative real-time polymerase chain reaction

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, California, USA) after 48 h of irradiation, and 2 μg of total RNA was used for the synthesis of first-strand cDNA with a reverse transcriptase kit (Invitrogen, Carlsbad, California,

Table 1. Clinical characteristics, radioreaction, and relationship with REGIA distribution.

Parameters	N (439)	REG1A expression (n,%)		χ2	P value
		Positive	Negative		
Age (year)				0.902	0.342
≤55	212	95(44.81)	117(55.19)		
>55	227	112(49.34)	115(50.66)		
Gender				0.174	0.677
Male	344	164(47.67)	180(52.33)		
Female	95	43 (45.26)	52 (54.74)		
Pathological type				0.505	0.477
WHO II	40	21(52.50)	19(47.50)		
WHO III	399	186(46.62)	213(53.38)		
T stage				18.124	0.000
T1–2	240	91(37.92)	149(62.08)		
T3–4	199	116(58.29)	83(41.71)		
N stage				6.527	0.011
N0–1	314	136(43.31)	178(56.69)		
N2	125	71(56.80)	54(43.20)		
N stage				2.837	0.092
N0	201	86(42.79)	115(57.21)		
N+	238	121(50.84)	117(49.16)		
Clinical stage				4.435	0.035
Stage I-II	125	49(39.20)	76(60.80)		
Stage III-Ⅳ	314	158(50.32)	156(49.68)		
Radiation dose to primary site				0.860	0.354
≤71Gy	216	97(44.91)	119(55.09)		
≥72 Gy	223	110(49.33)	113(50.67)		
Radioreaction				5.258	0.022
Radio-sensitive	380	171(45.00)	271(55.00)		
Radio-resistance	59	36(61.02)	23(38.98)		

USA). The mRNA level was measured by quantitative real-time polymerase chain reaction (qRT-PCR) with a Power SYBR Green qRT-PCR SuperMix (Invitrogen, Carlsbad, California, USA) on an ABI Prism 7500 HT sequence detection system (Applied Biosystems, Foster City, California, USA). The primers used for the qRT-PCR were as follows:
REGIA-F1:5'AACATGAATTCGGGCAACC;
REGIA-R1:5'AGGCCAATCCAGACATTGAA;
18s-F:5' CCTGGATACCGCAGCTAGGA;
18s-R:5' GCGGCGCAATACGAATGCCCC.

The internal control was 18S rRNA. The relative quantification of target mRNA expression was determined through the 2-ΔΔCt method. The amplification reactions were performed in triplicate for each examined sample.

Western blotting

After 48 h of irradiation, the cells were lysed in a Radio Immunoprecipitation Assay (RIPA) buffer (Sigma-Aldrich,St. Louis, Missouri, USA) containing protease and phosphatase inhibitors, and the protein concentration was measured using a Bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, Illinois, US). Equal amounts of protein lysates were electrophoretically separated on 10% sodium dodecyl sulfate-polyacrylamide gels for electrophoresis and transferred onto polyvinylidene fluoride membranes. The membranes were blocked with 5% non-fat dried milk for 1 h at 20°C. Then, they were incubated with primary antibodies (rabbit monoclonal antibodies against the REGIA antibody, dilution 1:500, Genetex, Illinois, USA) at recommended concentrations in Tris-buffered saline (TBS) and 0.1% Tween® 20 Detergent for 2 h at 20°C. After incubation with a horseradish peroxidase-conjugated secondary antibody (dilution 1:5,000, Rabbit Anti-Mouse IgG, Southern Biotech, Birmingham, AL, USA) for 1 h at 20°C, the protein bands were detected using an enhanced chemiluminescence reagent detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to the manufacturer's instructions. Glyceraldehyde-3-phosphate dehydrogenase served as an internal control for normalization. The amplification reactions were performed in triplicate for each examined sample.

Immunohistochemistry and evaluation of immunohistochemical staining

A total 439 pretreated NPC tissues, sliced consecutively, were available for immunohistochemistry according to the criteria above mentioned. This study examined REGIA expression by

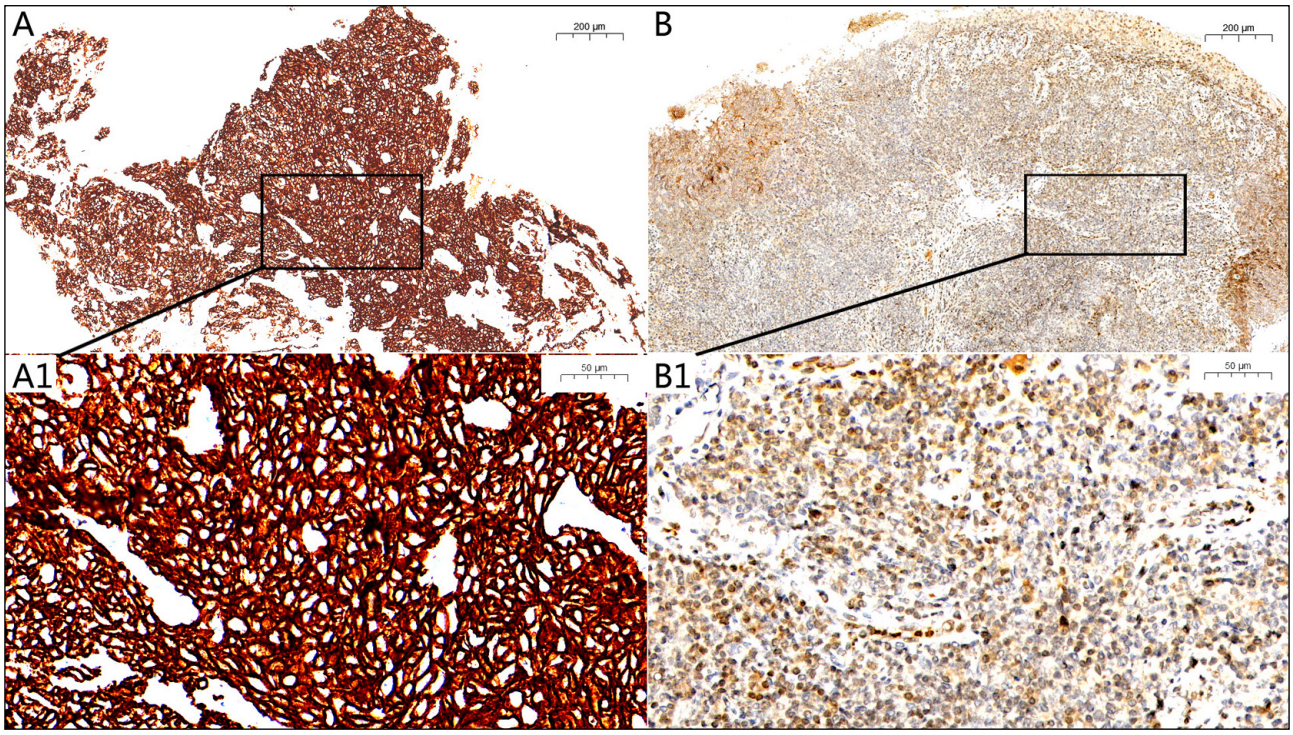

Fig. 1. Representative staining of regenerating gene IA (REGIA) in nasopharyngeal carcinoma tumors by immunohistochemistry (formalin-fixed paraffin-embedded 4-µm sections, streptavidin-peroxidase). (A): showing the expression positive of REGIA detection, 50×, staining in cytoplasm, and (A1) showing the higher magnification of 200× from the area of the box in (A); (B): showing the expression negative of REGIA detection, 50×, staining in cytoplasm, and (B1) showing the higher magnification of 200× from the area of the box in (B).

immunohistochemical staining according to a previous report (36). Briefly, the obtained biopsy tissue samples were immediately fixed in 10% formaldehyde for over 24 h, dehydrated with gradient alcohol, and embedded in paraffin. Then, 4-µm thick sections were obtained from the paraffin blocks. Subsequently, these sections were deparaffinized in xylene and rehydrated in an alcohol gradient. Finally, they were washed with phosphate-buffered saline (pH 7.4). Then, the sections were subjected to heat-induced antigen retrieval in a sodium citrate buffer (0.01 M, pH 6.0). This was followed by blocking of the endogenous peroxidase by immersing the slides in 250 ml of methanol that contained 2.5 ml of hydrogen peroxide solution for 30 min. The anti-REGIA rabbit monoclonal antibody (XM48, Ruifan, China) was incubated with the slices for 1 h at 20°C. This was followed by three separate 5 min washes with TBS and incubation with biotinylated anti-rabbit secondary antibody (SP-900 (general type), Zhongshan, China) for 30 min at 20°C. Subsequently, three more 5 min washes with TBS were performed, after which the sections were incubated with the streptavidin-biotin complex at 1:500 for 30 min at 20°C and then stained with 3, 3-diaminobenzidine. After counterstaining with hematoxylin, the slides were dehydrated and mounted for visualization. Rabbit IgG1 (ZA-0448, Zhongshan, China) served as a negative control. Batch-to-batch variation was assessed by choosing two sections with high and low REGIA expression and by running additional sections from these biopsy samples within each batch (*Fig. 1*).

The immunostaining results were evaluated and scored blindly by two independent pathologists, and a consensus was reached on any conflicting scores by discussion. The REGIA staining results were scored into four levels according to the percentage of cytoplasmic-positive tumor cells in 10 high-power fields for each slide, as follows: 0: less than 5%; 1: 6–25%; 2: 26–50%; and 3: more than 50%. Similarly, the staining intensity was assigned scores, which were as follows: 0: no staining, 1: weak staining, 2: moderate staining, and 3: strong staining. Two individual parameters were added, resulting in an immunoreactivity score (IRS) that ranged from 0 to 6. The cases with an IRS>4 were defined as positive expression, and the cases with an IRS≤4 were defined as negative expression (40).

Statistical analysis

The primary endpoint was progression-free survival (PFS), and the secondary endpoint was overall survival (OS). The duration of time to loco-regional recurrence was measured from the date of the start of radiotherapy until the failure of documented treatment. The patient's PFS was assessed from the start of treatment to the first defined event of failure, (*i.e.*, loco-regional recurrence and/or distant metastasis in patients who had completely responded to radiation therapy as well as the definite progression of disease in patients who had partially responded). The duration of OS was calculated from the start of radiation therapy until death due to any cause or until the date of the last follow-up visit for patients who were still alive.

These endpoints were analyzed and compared using the Kaplan-Meier method and the log-rank test. Univariate and multivariate Cox regression analyses were performed to determine the prognostic value of REGIA expression. A Student's t-test was used to compare mRNA and protein expression levels in cells, and the results were presented as the mean ± standard deviation. The differences in the clinicopathological parameters of positive REGIA expression

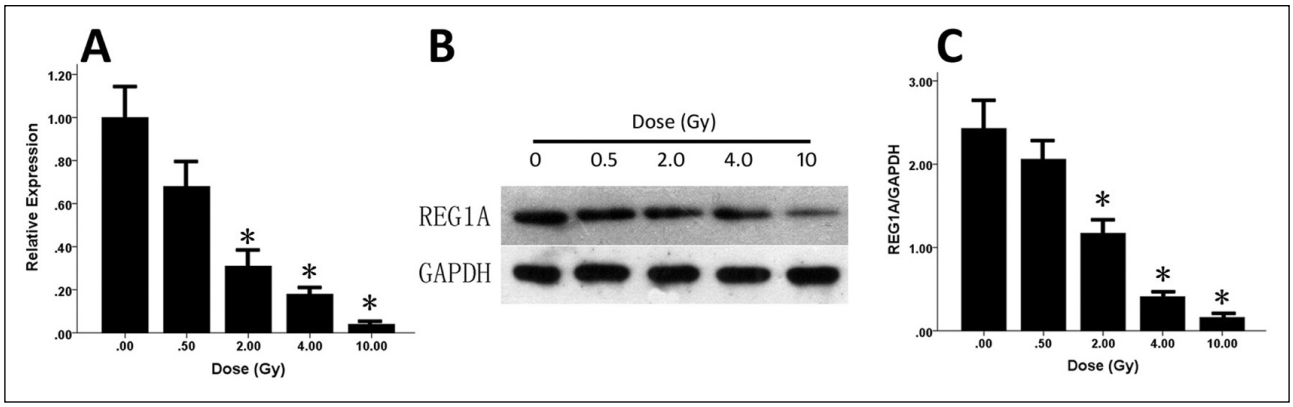

Fig. 2. Expression of regenerating gene IA (REGIA) in the nasopharyngeal carcinoma cell line of CNE1 at 48 h after radiation. (A): Quantitative real-time polymerase chain reaction detection showed that the mRNA expression levels of REGIA decreased gradually with the increase of radiation dose and presented lower levels of mRNA expression of REGIA at higher radiation doses (2, 4, and 10 Gy, respectively) than that at lower doses (0 and 0.5 Gy, respectively). Data represent three independent experiments, mean ± standard deviation (All P<0.05, respectively); (B): Western blot analysis showed that the expression of REGIA protein levels decreased at different doses of radiation (0.5, 2, 4, and 10 Gy, respectively); (C): Western blot analysis showed that the expression of REGIA protein levels at higher radiation doses (2, 4, and 10 Gy respectively) was lower than that at lower doses (0 and 0.5 Gy, respectively). (All P<0.05, respectively). The protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. The results are shown as an expression relative to GAPDH and are the mean ±standard deviation of three trials.

were assessed by the $\chi 2$ test, Pearson's chi-square test, or Fisher's exact test. The correlation between REGIA protein expression and its response to radiotherapy was analyzed by a Spearman's rank correlation test. The statistical 2-sided test was conducted by SPSS 19.0 software.

RESULTS

Follow-up data

The median follow-up duration was 39.6 months (range: 7.8–99.6 months) for all patients. Diagnostic criteria for recurrence:

1. Time criteria: the cut-off point is 6 months after the end of clinical treatment. Lesions occurring after 6 months were identified as recurrent, and lesions occurring within 6 months were identified as uncontrolled. Both recurrent and uncontrolled cases were counted as recurrent in clinical data.

2. Inspection method: CT scan (or with enhanced scan), electronic fibroscopy and tumor tissue biopsy, *etc*.

3. Lesion region: includes primary lesion site, regional lymph nodes, and distant organs. During the follow-up period, 55 patients developed loco-regional recurrence or distant metastasis, and the recurrence rate was 13.4% (59/439). It should be noted that only 5 cases were confirmed by biopsy, while the rest were confirmed by CT and fiber endoscopy in this group of cases.

REGIA expression in nasopharyngeal carcinoma cells and tissues

The REGIA mRNA expression levels were evaluated by qRT-PCR (*Fig. 2A*), and Western blotting was performed to evaluate protein expression (*Fig. 2B* and *2C*). The results showed that CNE1 cells treated with increasing radiation doses had decreasing levels of REGIA mRNA and protein expressions when compared to cells that underwent no treatment, and the difference was statistically significant (P=0.002 versus P=0.004, respectively) (*Fig. 2A-2C*).

The rates of positive REGIA expression from stage T1 to stage T4 were 33.3%, 40.3%, 45.5%, and 68.5%, respectively, showing significant differences between each stage from T1 to T3 and T4 stage tumors as well as T1–T2 stage and T3–T4 stage tumors. These results indicate that patients with positive REGIA expression had a higher T stage than those with negative REGIA expression. Significant differences also existed in the clinical stage and lymph node metastasis status (P<0.05, respectively). Interestingly, significant differences were observed between N0–1 and N2. These results may suggest that REGIA expression significantly enhances cell proliferation in patients with advanced NPC. No significant differences existed in age, gender, and WHO classification (*Table 1*).

Radiosensitivity assessment and association with REGIA expression

Of the 439 patients, 380 patients were radiosensitive; in this group, 171 patients were REGIA-positive, and the remaining 209 were REGIA-negative. Of the 439 patients, 59 were radioresistant, and, of those, 36 cases were positive, and 23 were negative.

The rate of positive REGIA expression in the radiosensitive patients versus the radioresistant patients was 45.0% (171/380) versus 61.0% (36/59), respectively, showing significant differences (P=0.022, *Table 1*). No difference was observed in the radiation dose of the primary tumor (P=0.354, *Table 1*). A Spearman's analysis showed that positive REGIA protein expression had a negative correlation with the radiosensitivity of NPC cells (r= –0.109, P=0.047). Additionally, REGIA expression could discriminate NPC patients' responses to radiation.

Effect of REGIA expression on survival

The five-year OS and PFS rates were 50.4% and 50.1%, respectively. Similarly, the five-year OS and PFS rates were 34.0% and 34.5%, respectively, for patients with positive REGIA expression and 63.4% and 62.0%, respectively, for patients with negative REGIA expression. A significant

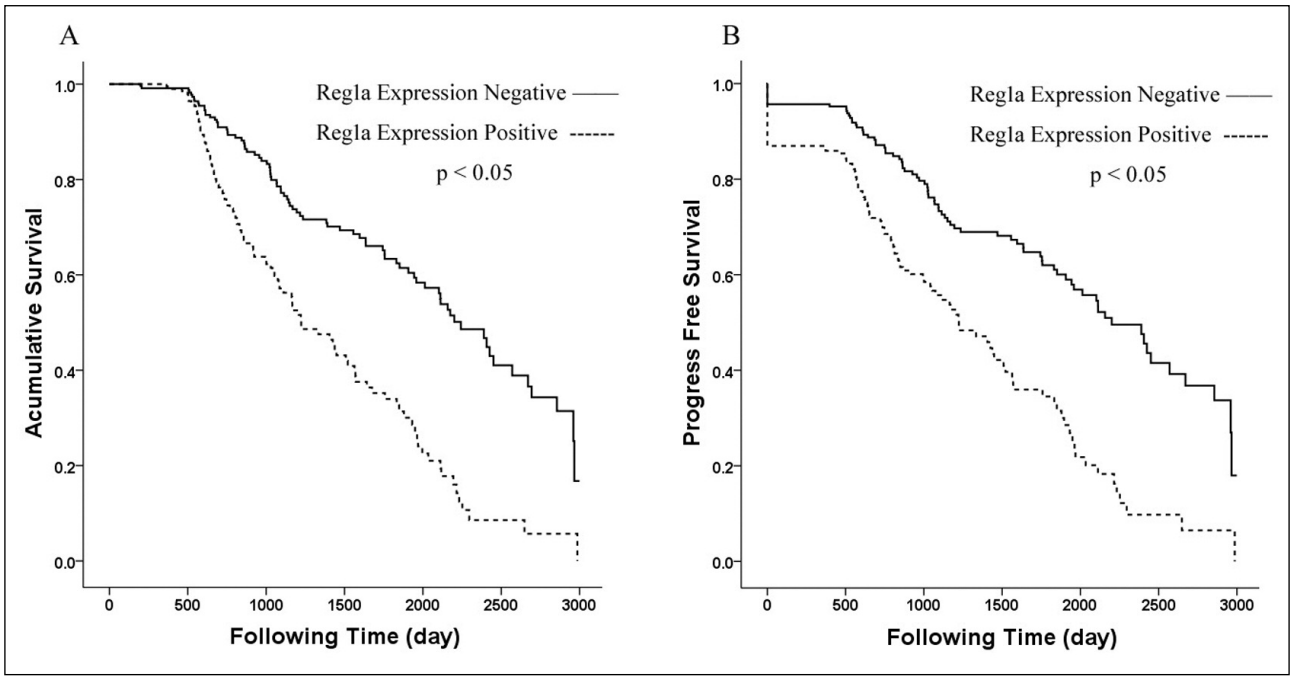

Fig. 3. The Kaplan-Meier survival curve and log-rank test for nasopharyngeal carcinoma (NPC) patients stratified according to the positive or negative REGIA expression. (A): The overall survival (OS) curve of NPC patients with different REGIA expressions and the five-year OS rate was significantly different between NPC patients with negative expression (63.4%) and positive expression (34.0%) (P<0.001; log-rank test); (B): The progression free survival (PFS) curve of NPC patients with different REGIA expressions and the five-year PFS rate was significantly different between NPC patients with negative expression (62.0%) and positive expression (34.5%) (P<0.001; log-rank test).

difference was observed based on the log-rank test (P<0.001) (*Fig. 3A-3B*).

The following variables were entered into the Cox regression model: the irradiation dose to the neck, the irradiation dose to the primary nasopharyngeal site, gender, age, TNM stage, T stage, N stage, treatment effect, and REGIA expression. The results revealed that positive REGIA expression was an independent prognostic factor of OS (hazard ratio (HR)=2.139, 95% confidence interval (CI)=1.556–2.940, P<0.001) and PFS (HR=1.958, CI=1.424–2.693, P<0.001), (*Table 2*).

DISCUSSION

Radical radiation is considered the most effective treatment for NPC. However, patients with the same stage, radiation dose, and grade of tumor have varied prognoses. Therefore, the ability to assess pretreatment radiation sensitivity and provide a personal therapeutic schedule is highly desired.

In fact, the intrinsic radiosensitivity determinant in tumors is based on the functional status of the gene or genome and/or its expressing protein, and this, in turn, regulates the proliferation and/or apoptosis of cancer cells. Collected evidence implies that some genes can accurately reflect radiosensitivity against cancer (41-43), and REGIA expression was previously shown to improve radiotherapy sensitivity in esophageal squamous cell carcinomas (28). Therefore, this study inferred that REGIA expression might be related to radiosensitivity and could be a predictor in NPC.

Few data are available to determine the clinical implication of REGIA expression in tumors; in esophageal cancer, it was shown that REGIA overexpression implied chemoradiotherapy sensitivity (26-28, 30). In this current study, REGIA expression correlated with radiosensitivity and was an adverse prognostic factor for OS and PFS for NPC patients who received radiation alone.

This study's findings revealed that REGIA mRNA and protein expression levels differed depending on the varied radiation doses in CNE1 cells. The relevant REGIA expression levels declined as radiation doses increased (P<0.05), which may indicate that REGIA functions are strongly associated with radiation reactions.

In exploring the underlying mechanism of REGIA expression on radiosensitivity, Sekikawa *et al*. confirmed that interferon (IFN)-gamma and IL-6 improved REGIA gene expression and its promoter activity. The REGIA protein promoted cell growth and cell resistance to H_2O_2-induced apoptosis by regulating Akt phosphorylation and Bcl-xL expression in gastric adenocarcinoma cell (AGS) cells (21). In addition, Wakita *et al*. verified that REGIA can affect the radiosensitivity of cancer *via* mediation of the JNK and ERK pathways (27). However, the effect of REGIA expression on the radiosensitivity of NPC has yet to be clarified, and the pathway that regulates the reaction of NPC radiation needs to be explored.

Hayashi *et al*. found that REGIA overexpression demonstrated better chemoradiosensitivity and had a more favorable prognosis in patients with squamous cell esophageal carcinoma (7, 26). Transfection with REGIA in TE-5 and TE-9 cells led to strong expression of REGIA mRNA and protein and a significant increase in both chemosensitivity and radiosensitivity (28). The mechanisms underlying this study's results (*i.e*., that REGIA expression levels differed depending on the varied radiation doses and that REGIA regulates radiosensitivity levels) may include the following: 1) interaction with DNA repair genes; 2) direct involvement in DNA repair; 3) cell cycle regulation, and 4) other unknown signal paths.

Table 2. Multivariate Cox regression analyses of overall survival and progression-free survival in patients with nasopharyngeal carcinoma.

Parameters	Overall survival (OS)					
	B	**SE**	**Wald**	**P**	**Exp (B)**	**95.0% CI**
Age	0.008	0.153	0.003	0.957	1.008	0.747–1.362
Gender	–0.237	0.193	1.516	0.218	0.789	0.541–1.151
Pathology	–0.253	0.236	1.152	0.283	0.777	0.489–1.232
T stage	0.283	0.117	5.830	0.016	1.327	1.055–1.670
N stage	0.212	0.149	2.029	0.154	1.236	0.923–1.655
Clincal stage	0.045	0.150	0.088	0.767	1.046	0.779–1.404
Radiotherapy action	0.197	0.231	0.728	0.393	1.218	0.775–1.914
REG1A expression	0.760	0.162	21.937	<0.001	2.139	1.556–2.940
Primary radiation doses	–0.106	0.148	0.509	0.475	0.900	0.673–1.202
Parameters	**Progression-free survival (PFS)**					
	B	**SE**	**Wald**	**P**	**Exp (B)**	**95.0% CI**
Age	0.081	0.152	0.285	0.593	1.085	0.805–1.462
Gender	–0.216	0.193	1.256	0.262	0.806	0.552–1.175
Pathology	–0.127	0.235	0.293	0.588	0.880	0.555–1.397
T stage	0.185	0.120	2.386	0.122	1.203	0.952–1.521
N stage	0.157	0.150	1.092	0.296	1.170	0.872–1.571
Clincal stage	0.127	0.151	0.701	0.402	1.135	0.844–1.528
Radiotherapy action	12.873	52.924	0.059	0.808	389570.273	0.000–4.364E50
REG1A expression	0.672	0.163	17.096	<0.001	1.958	1.424–2.693
Primary radiation doses	0.055	0.147	0.140	0.708	1.057	0.792–1.410

This study explored the clinical relationship between REGIA expression levels and radiosensitivity in NPC patients. The results showed the existence of a significantly higher REGIA-positive expression rate in radioresistant patients ($P<0.05$). Further validation indicated that pretreated positive REGIA expression levels are negatively associated with NPC radiosensitivity ($P=0.047$). Similar to its function in marking radiosensitivity in squamous cell esophageal cancer (8), REGIA acted as a marker for assessing pretreatment radiosensitivity for NPC.

This study's results indicated that REGIA-positive expression levels increased with a higher T stage, possibly suggesting that REGIA enhances the local invasion of NPC, consistent with a previous study (36). Interestingly, it was also observed that REGIA expression was positively associated with lymph node metastasis when comparing N0–1 with N2 rather than N0 with N1–2. Thus, it was speculated that REGIA might act as a 'molecular accelerator' in the cancer cell proliferation of NPC, which was in line with previous study results (19). This explains why a higher REGIA-positive expression rate was observed in advanced loco-regional lesions and radioresistant patients. Further research is warranted to discover the underlying mechanisms that regulate proliferation, such as the interaction between REGIA and microRNAs, which have confirmed that micro-RNA can regulate the proliferation, migration and invasion of nasopharyngeal carcinoma cells in recent studies (44).

These findings indicate the presence of a radiosensitivity determinant, and REGIA expression levels served as a biomarker to screen the intrinsic radiosensitivity of NPC cells. Therefore, any available means to regulate the functional status of REGIA, such as IFN-gamma, IL-6, JNK, or ERK, could act as a target for therapeutic intervention and could improve the tumor's radiosensitivity and the patient's clinical outcome.

This study's data showed that positive REGIA expression was a strong predictor of NPC prognosis. Higher levels of REGIA predicted adverse survival when compared with lower expression, consistent with a previous study (36), similar to the functional activities of REGIA in lung, breast, gastric, and colorectal cancers (13, 14, 29, 34, 37). Univariate and multivariate regression analyses suggested that REGIA expression was an independent predictor of OS and PFS in NPC patients. The pretreatment determination of REGIA expression levels serves as biomarkers for assessing the prognosis of patients with NPC. It should be pointed out that there are currently few studies on the relationship between REGIA expression and head and neck tumors, especially NPC. Data still need to be accumulated to verify and establish the guiding role of REGIA in the assessment of the radiation response of NPC cells and the prognosis of patients diagnosed with NPC.

Most patients with negative REGIA expression are radioresistant, and radiation therapy alone was less effective for these patients in the present study. This indicates that these patients might have a better prognosis with an aggressive course of treatment that includes chemotherapy, targeted molecular therapy, and immunotherapy along with radiotherapy. In addition, rigorous follow-up of these patients should be performed. On the other hand, some patients with REGIA-positive expression also showed radioresistance, indicating that other factors were involved in the regulation of radiotherapy sensitivity, such as the action and interaction of other genes in the REGIA-related signaling pathway, the differences in the microenvironment determined by tumor heterogeneity, the signaling network formed by mRNA with or without genes, and others, all of which need further research.

In addition, it should be pointed out that radiotherapy with chemotherapy is routinely used in the treatment of advanced NPC. Therefore, additional research is necessary to study the relationship between REGIA expression and chemotherapy response as well as the role of REGIA in the mechanism of chemotherapy sensitivity; findings related to these issues will help to establish the predictive role of REGIA in the treatment response of NPC.

Intrinsic radiosensitivity and the prognosis of cancer were influenced by clinical staging, radiation dose, tumor differentiation degree, and other factors; however, there is no doubt that the presence of REGIA mRNA and protein are key factors and act as effective markers for radiosensitivity and survival prediction in NPC. More attention should be paid to the relevant REGIA signaling pathways and mechanisms to screen for and reveal other relevant molecular mechanisms. These additional mechanisms may be useful because combining genes or proteins together improves the prediction accuracy.

In conclusion, radiation can affect REGIA expression. This study's results revealed that the expression of REGIA was correlated with NPC radiosensitivity and the prognosis of patients who received radiation as their sole therapy. The expression of REGIA also acts as a potential molecular marker that is worthy of further exploration in future studies.

Abbreviations: Bcl-xL, B-cell lymphoma-extra-large; CI, confidence interval; CR, complete response; ERK, extracellular signal-regulated kinase; IRS, immunoreactivity score; NA, no assessment; NPC, nasopharyngeal carcinoma; OS, overall survival; PD, progressive disease; PFS, progression-free survival; PR, partial response; qRT-PCR, quantitative real-time polymerase chain reaction; REGIA, regenerating Gene IA; SD, stable disease; STAT3, signal transduction and activation of transcription 3.

Availability of data and material: All data generated or analysed during this study are included in this article. Further enquiries can be directed to the corresponding author.

Authors' contributions: HJX and HBC conceived the idea, XDC and HXS conceptualised the study. YZD collected the data. YFH and LLD analysed the data. HJX, JHL and drafted the manuscript and reviewed the manuscript. All authors read and HJX approved the final draft. All authors contributed equally to this work All participants signed a document of informed consent.

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