

URG4 expression is a novel prognostic factor for the progression of nasopharyngeal carcinoma and overall survival of patient

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Abstract: URG4, a novel oncogene, is involved in the development and progression of various tumors. This study investigated the clinicopathological significance of URG4 in nasopharyngeal carcinoma (NPC). We used five NPC tissues and adjacent normal nasopharyngeal tissues to determine URG4 expression and found that URG4 was upregulated in NPC tissues. Immunohistochemistry analysis found URG4 was expressed positively in 97.1% (99/102) of NPC samples and highly expressed in 41.2% (42/102) of NPC samples. Its level was positively correlated with advancing clinical stage. Kaplan–Meier analysis with the log-rank test found that patients with high URG4 expression had poor outcome and patients with low URG4 expression had better survival. Statistical analysis showed that there was a significant correlation between URG4 expression and clinical stage, larger tumor size, and lymph node involvement. Cox-regression analysis showed that URG4 expression could serve as a prognostic factor for NPC patients. In summary, this study showed that URG4 was upregulated in NPC tissues, patients with high URG4 expression had poor outcome, and URG4 was found to be a valuable biomarker for NPC progression.

Keywords: URG4, nasopharyngeal carcinoma, prognostic factor

Introduction

Nasopharyngeal carcinoma (NPC) is one of the most common malignant neoplasms in southern China – the incidence in Guangdong Province is about 100-fold higher compared to Europe and North America. Some susceptibility loci and oncogenes have been demonstrated to promote or suppress NPC progression, and in the past few decades, they have been found to serve as prognostic factors and therapy targets; for example, TNFRSF19, CDKN2A-CDKN2B, and MDS1-EV11 are some newly identified susceptibility loci.¹ BMI1 is a new oncogene for NPC and promotes epithelial–mesenchymal transition (EMT) Mechanistic analyses have found that it binds to tumor suppressor PTEN directly and inhibits its activity to activate PI3K/Akt/GSK-3 β signaling pathway. This signaling can increase the stability of Snail that facilitates EMT. Snail also suppresses PTEN expression, PTEN, and PI3K/Akt/GSK-3 β and forms a positive feedback loop.² But the regulatory mechanism of the development and progression of NPC has not been understood well, and hence the survival of patients with NPC is still poor. However, early diagnosis is critical for NPC treatment and identification of useful biomarkers is essential for NPC prognosis.

URG4, a novel oncogene, promotes the progression of various tumors. For example, it is a natural product of hepatitis B \times antigen (HB \times Ag), which participates in

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hepatocarcinogenesis.³ URG4 is upregulated in hepatocellular carcinoma (HCC) tissues. Statistical analyses suggest that its expression is significantly correlated with clinical stages and poor survival of HCC patients. Functional analyses have found that URG4 not only activates Akt signaling pathway, and suppresses cell cycle inhibitors p27 and p21 to promote cell proliferation,^{4,5} but also activates NF- κ B pathway to facilitate angiogenesis.⁶ URG4 expression is higher in ovarian cancer tissues and hence can serve as a new prognostic marker for ovarian cancer.^{7,8} Apart from HCC and ovarian cancer, URG4 also plays critical roles in gastric cancer,⁹ bladder cancer,¹⁰ glioblastoma multiforme,¹¹ non-small-lung cancer,¹² medullary thyroid cancer,¹³ neuroblastoma,¹⁴ prostate cancer,¹⁵ and leukemia.¹⁶ But the role of URG4 in NPC has not been reported yet.

In this study, we determined URG4 expression in NPC tissues and adjacent normal nasopharyngeal tissues and analyzed the relationship between URG4 expression and various clinicopathologic characteristics. Finally we used Cox-regression analysis to evaluate whether URG4 can serve as a prognostic marker for NPC patients.

Materials and methods

Patients and tissue specimens

A total of 102 paraffin-embedded NPC samples were histopathologically diagnosed in 1998–2003 at the Guangzhou First People's Hospital, Guangzhou Medical University, Guangdong, People's Republic of China. These clinical materials were used for research purposes after obtaining patient's written consent. This study was approved by the Institutional Research Ethics Committee of the Guangzhou First People's Hospital. Clinicopathological information of these samples is shown in Table 1. The clinical stages of all patients were defined according to the Chinese 1992 staging system.¹⁷

Five NPC specimens (T) and adjacent normal nasopharyngeal specimens were also obtained from the Guangzhou First People's Hospital, Guangzhou Medical University. These samples were embedded using paraffin and were stored at 4°C until use.

Immunohistochemistry

Immunohistochemistry (IHC) was performed according to the previously described method by Cao et al.¹⁸ Briefly, all paraffin-embedded specimens were cut into 4 μ m sections and deparaffinized with xylenes. They were then rehydrated with graded ethanol to distilled water. Antigen retrieval was performed by submerging sections into ethylene diamine tetraacetic acid buffer (pH 8.0) and heating in a microwave oven. A total of

Table 1 Clinicopathological characteristics of NPC patient samples

	Number of cases (%)
Sex	
Male	75 (73.5)
Female	27 (26.5)
Age, years	
\geq 45	53 (52.0)
<45	49 (48.0)
Clinical stage	
I	4 (3.9)
II	27 (26.5)
III	50 (49.0)
IV	21 (20.6)
T classification	
T1	23 (22.5)
T2	46 (45.1)
T3	24 (23.5)
T4	9 (8.8)
N classification	
N0	12 (11.8)
N1	32 (31.4)
N2	48 (47.1)
N3	10 (9.8)
M classification	
No	99 (97.1)
Yes	3 (2.9)

Abbreviations: NPC, nasopharyngeal carcinoma; T, tumor; N, nodes; M, metastasis.

0.3% H₂O₂ was used to treat sections for 15 minutes to block endogenous peroxidase after being heated, and then the sections were incubated with 1% bovine serum albumin to block nonspecific binding and then incubated with anti-URG4 antibody (1:150, HPA020134, Sigma-Aldrich Co., St Louis, MO, USA) overnight at 4°C. After washing with phosphate-buffered saline, the sections were incubated with biotinylated secondary antibody, followed by further incubation with streptavidin–horseradish peroxidase at 37°C for 30 minutes. The sections were immersed in 3,3'-diaminobenzidine for 10 minutes and counterstained with 10% Mayer's hematoxylin, dehydrated, and mounted in crystal mount. For negative controls, the primary antibody was replaced by normal goat serum.

The results of staining were scored independently by three pathologists blinded to clinical outcome according to the proportion of positively stained tumor cells and the intensity of staining. The proportion of positively stained tumor cells was scored as follows: 0 (no positive tumor cells), 1 (<20% positive tumor cells), 2 (20%–50% positive tumor cells), 3 (50%–80% positive tumor cells), and 4 (>80% positive tumor cells). The intensity of staining was graded as follows: 0 (no staining), 1 (weak staining, light yellow), 2 (moderate staining, yellow brown), and 3 (strong staining, brown). The staining index (SI) was calculated as the product

of the staining intensity score and the proportion of positive tumor cells score, resulting in scores of 0, 1, 2, 3, 4, 6, 8, 9, and 12. Cutoff values for high and low expression of URG4 were chosen according to the measurement of heterogeneity using the log-rank test with respect to overall survival. The optimal cutoff for high expression of URG4 was identified as an SI score ≥ 4 and the cutoff for low expression of URG4 was identified as an SI score ≤ 3 .

Statistical analysis

All statistical analyses were performed using the SPSS 19.0 statistical software package. Chi-square and Fisher's exact tests were used to analyze the relationship between URG4 expression and clinicopathologic parameters. Bivariate correlations between variables were calculated by Spearman's rank correlation coefficients. Survival curve was plotted using Kaplan–Meier method and compared by the log-rank test. Univariate and multivariate Cox-regression analyses were used to analyze the significance of various variables for survival. A value of $P < 0.05$ (two tailed) was considered significant in all cases.

Results

URG4 is upregulated in NPC tissues

To investigate the role of URG4 in NPC, we determined the expression of URG4 in the NPC tissues and adjacent normal nasopharyngeal tissues of five patients by IHC. We found that URG4 was expressed only in primary NPC tissues and no specific URG4 staining was observed in adjacent normal nasopharyngeal tissues (Figure 1). These suggested that URG4 was upregulated in NPC tissues.

URG4 expression is positively correlated with clinical aggressiveness of NPC

We determined URG4 expression in 102 paraffin-embedded archival NPC tissues using IHC. These samples included four stage I tumors, 27 stage II tumors, 50 stage III tumors, and 21 stage IV tumors. URG4 was detected in 99 of 102 (97.1%) cases. Its expression was high in 42 (41.2%) cases and low in 60 (58.8%) cases (Table 2). We also found that URG4 was mainly located in cytomembrane (Figure 2). We further investigated URG4 expression in different clinical stages and the detailed data are as follows: 0% for stage I (0/4), 6.9% for stage II (2/27), 46% for stage III (23/50), and 81% for stage IV (17/21) (Table 3); this suggests that URG4 expression was positively correlated with advancing clinical stage (Figure 2).

We analyzed the association between URG4 expression and the patient's clinicopathologic characteristics. As shown in Table 3, we found that URG4 expression was significantly correlated with clinical stage, larger tumor size (T classification), and lymph node involvement (N classification). But whether URG4 expression was significantly correlated with distant metastasis (M classification) is not clear because different P -values were calculated according to different statistical methods ($P=0.036$ using chi-square test and $P=0.067$ using Fisher's exact test). However, there was no significant correlation between URG4 expression, sex, and age. We further analyzed the correlation between URG4 expression and clinical stage, larger tumor size, lymph node involvement, and distant metastasis using Spearman's correlation analysis and found that URG4 expression was positively correlated with these clinicopathologic characteristics (Table 4).

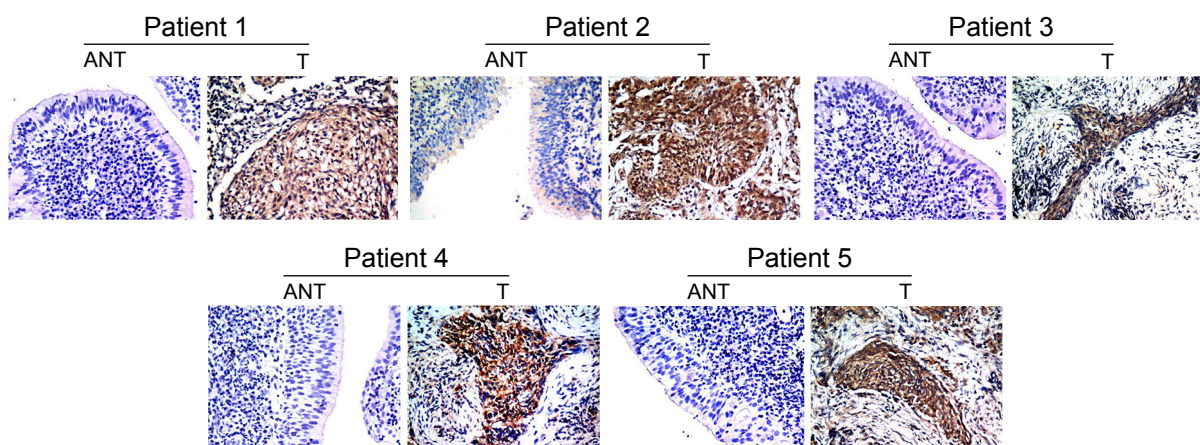


Figure 1 Upregulation of URG4 in NPC tissues.

Note: URG4 expression in five pairs of matched NPC tissues analyzed by immunohistochemistry. Magnification 200x.

Abbreviations: NPC, nasopharyngeal carcinoma; T, tumor; ANT, adjacent normal tissues.

Table 2 The expression of URG4 in NPC tissues

Expression of URG4	Number (%)
Negative	3 (2.9)
Positive	99 (97.1)
Low expression	60 (58.8)
High expression	42 (41.2)

Abbreviation: NPC, nasopharyngeal carcinoma.

High URG4 expression is associated with an unfavorable prognosis of NPC

We investigated the correlation between URG4 expression and patient's survival using Kaplan–Meier analysis with the log-rank test. We found that URG4 expression was significantly negatively correlated with the overall survival of NPC patients, patients with low expression of URG4 had better outcome, and patients with high expression of URG4 had poor survival (Figure 3).

We used univariate Cox-regression analysis to determine the clinical significance of various prognostic factors that might influence the survival and tumor progression in NPC patients. The results showed that clinical stage, T classification, and URG4 expression were significant poor prognostic factors. Multivariate Cox-regression analysis revealed that clinical stage, T classification, and URG4 expression were independent prognostic factors for patients with NPC (Table 5). Thus, these results suggested that URG4 expression has a significant correlation with the prognosis of NPC and could serve as a biomarker for the prognosis of NPC patients.

Discussion

In this study, we found that URG4 was overexpressed in NPC tissues and its expression was positively correlated

with advancing clinical stage. Statistical analysis revealed that URG4 expression significantly correlated with NPC progression and patients with high URG4 expression had poor clinical outcome. Therefore, it can function as an unfavorable prognostic factor.

New valuable prognostic factors are important to develop novel therapeutic strategies. Recently, many prognostic factors, such as centromere protein H (CENP-H)¹⁷ and MMP9,¹⁹ have been found. To estimate cancer progression and the outcomes of NPC patients, more biomarkers have to be discovered. We used a cohort of 102 patients' samples to determine the relationship between URG4 expression and patient survival. We found that patients with high URG4 expression had poor survival. To demonstrate whether URG4 could serve as an indicator of NPC progression, we analyzed the correlation between URG4 expression and clinicopathologic parameters; we found that with the progression of NPC, the URG4 expression was upregulated gradually. This suggested that URG4 expression was significantly positively correlated with advancing clinical stage. We also found that there was a significant correlation between clinical stage, larger tumor size, and lymph node involvement. These suggested that URG4 was a biomarker of NPC progression. Univariate and multivariate Cox-regression analyses showed that patients with high URG4 expression had poor prognosis. These results emphasize that URG4 is a novel biomarker for NPC and it can be combined with other identified biomarkers to choose appropriate therapy method, such as adjuvant chemotherapy, radiotherapy, or chemotherapy, and to predict the clinical stage and survival rate.

URG4 localization is different in different kinds of tumors. In ovarian cancer and HCC, URG4 mainly is expressed in cytoplasm,^{4,8} whereas in cervical cancer, URG4

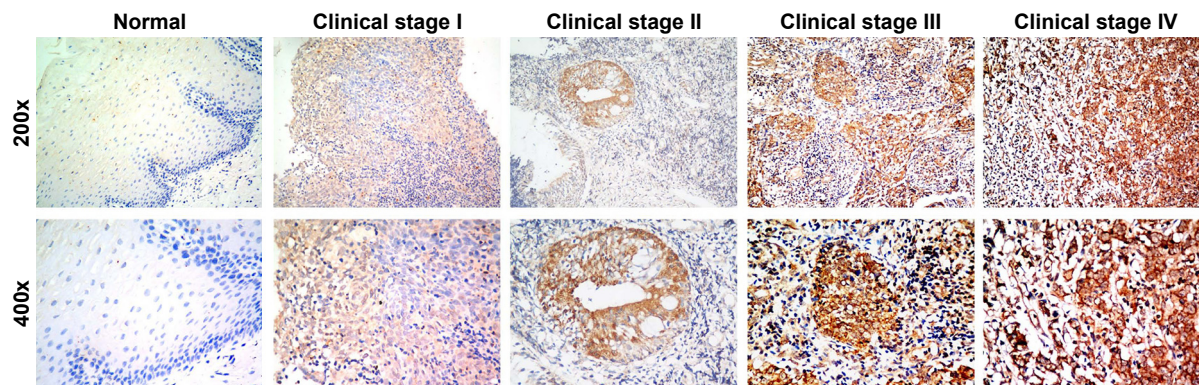


Figure 2 URG4 expression is positively correlated with advancing clinical stage.

Note: Representative images of URG4 expression in nasopharyngeal tissues and NPC tissues at different clinical stages.

Abbreviation: NPC, nasopharyngeal carcinoma.

Table 3 The correlation between URG4 expression and clinicopathologic characteristics of NPC

Characteristics	URG4		Chi-square test; P-value	Fisher's exact test; P-value
	Low or none (number of cases)	High (number of cases)		
Sex				
Male	41	34	0.155	0.178
Female	19	8		
Age (years)				
≥45	29	24	0.381	0.425
<45	31	18		
Clinical stage				
I	4	0.000	0.000	0.000
II	25	2		
III	27	23		
IV	4	17		
T classification				
T1	21	2	0.001	0.000
T2	26	20		
T3	11	13		
T4	2	7		
N classification				
N0	10	2	0.000	0.000
N1	26	6		
N2	22	26		
N3	2	8		
M classification				
No	60	39	0.036	0.067
Yes	0	3		

Abbreviations: NPC, nasopharyngeal carcinoma; T, tumor; N, nodes; M, metastasis.

mainly is found in plasma membrane.⁷ We found that presence of URG4 in cytomembrane in NPC conditions facilitated clinical diagnosis.

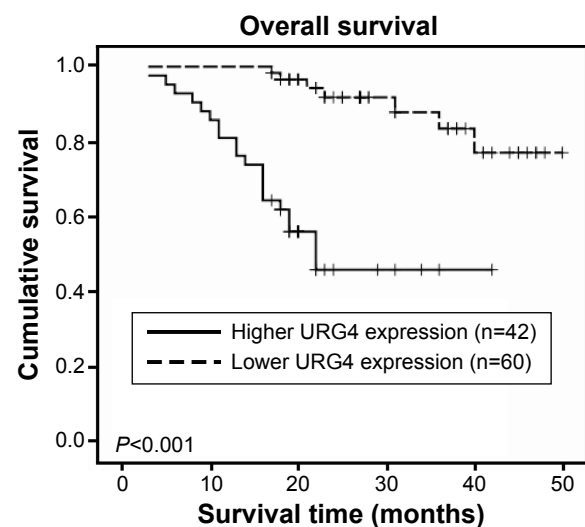
Previous reports have shown that URG4 activates Akt and NF- κ B pathway in HCC.^{4,6} Akt pathway⁷ plays critical role in survival, glucose uptake, angiogenesis, proliferation, and metabolism. It inhibits Wee1, Myt1, p27, p21, and cyclin D1 and suppresses the progression of cell cycle. It also inhibits FOXO1, FOXO3a, and Bad, and meanwhile activates MDM2 to regulate apoptosis.²⁰ Akt has been demonstrated to regulate EMT in NPC.² NF- κ B pathway regulates tumor cellular proliferation, apoptosis, angiogenesis, and metastasis.²¹ Akt² and NF- κ B pathway²² regulate proliferation, metastasis, and

Table 4 Spearman correlation analysis between URG4 and clinical pathologic factors

Variables	URG4 expression level	
	Spearman's correlation	P-value
Clinical staging	0.530	0.000
T classification	0.388	0.000
N classification	0.410	0.000
M classification	0.208	0.036

Abbreviations: T, tumor; N, nodes; M, metastasis.

apoptosis of NPC. There was a significant positive correlation between URG4 expression and clinical stage, larger tumor size, lymph node involvement, and distant metastasis, which suggested that URG4 might regulate proliferation and metastasis of NPC. We speculated that URG4 might regulate

**Figure 3** Kaplan–Meier curves with log rank test.

Note: The overall survival is poor in patients with high URG4 expression.

Table 5 Univariate and multivariate analyses of various prognostic parameters in patients with NPC Cox-regression analysis

	Number of patients	Univariate analysis		Multivariate analysis		
		P-value	Regression coefficient (SE)	P-value	Relative risk	95% Confidence interval
Clinical stage						
I	4	<0.001	1.926 (0.355)	<0.001	4.311	1.946–9.549
II	27					
III	50					
IV	21					
T classification						
T1	23	<0.001	1.113 (0.236)	0.031	1.701	1.051–2.754
T2	46					
T3	24					
T4	9					
Expression of URG4						
Low expression	60	<0.001	2.013 (0.457)	0.003	4.169	1.620–10.727
High expression	42					

Abbreviations: NPC, nasopharyngeal carcinoma; T, tumor; SE, standard error.

proliferation and metastasis through Akt pathway or NF- κ B pathway. These findings suggested that URG4 might be a novel target for NPC therapy. But the role of URG4 in NPC should be studied in depth.

Conclusion

In conclusion, our study suggested that URG4 was upregulated in NPC tissues and its level showed a significant correlation with clinical stage, larger tumor size (T classification), and lymph node involvement (N classification). Patients with high URG4 level had poor outcome and URG4 was found to be an independent prognosis factor for NPC patients.

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Disclosure

The authors report no conflict of interest in this work.

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