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ORIGINAL RESEARCH

Expression and Prognostic Value of Lgr5 in Patients with Recurrent Nasopharyngeal Carcinoma

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Background: As a cancer stem cells (CSCs) surface marker, Lgr5 plays an important role in the signal transduction of cancer cells and is a potential biomarker for cancer diagnosis and prognosis. However, the expression and prognostic value of Lgr5 in recurrent nasopharyngeal carcinoma (rNPC) remains ambiguous.

Materials: We used RNA sequencing to screen differentially expressed mRNAs in eleven specimens of rNPC tissues and five fresh adjacent normal tissue samples and the CSC marker, Lgr5, was identified. The expression level of Lgr5 in rNPC samples was also detected by immunohistochemistry and Western blot assay. The chi-square test was used to analyze the relationship between the clinicopathological variables and the immunostaining of Lgr5. The Log-rank method was used for prognosis analysis. The Cox regression model was used for univariate and multivariate analysis.

Results: Significantly elevated expression of Lgr5 in the rNPC tissues was observed compared to the normal tissues using RNA sequencing, Western blot and immunohistochemistry. The expression of Lgr5 was significantly correlated with the T stage (P=0.014). High Lgr5 expression (P=0.007), tumor necrosis (P=0.013) and WHO type II (P=0.043) in rNPC patients exhibited worse overall survival (OS). Lgr5 expression was proved to be an independent risk factor for OS (P=0.035) in multivariate analyses, and had promising predictive value for survival and recurrence in rNPC patients (area under the ROC curve: 0.711 and 0.665, P=0.017 and 0.028, respectively).

Conclusion: Lgr5 as a CSC marker is a promising therapeutic target and could be employed to predict the survival prognosis of rNPC patients.

Keywords: recurrent nasopharyngeal carcinoma, cancer stem cell, Lgr5, survival

Introduction

Nasopharyngeal carcinoma (NPC) is a malignant epithelial tumor that originates from the lining of the nasopharyngeal mucosa. In accordance with the International Agency for Research on Cancer, the global geographical distribution of NPC is extremely unbalanced; more than 70% of the new cases occur in East and Southeast Asia. The standardized incidence rate was 3.0 per 100,000 in China and 0.4 in predominantly white populations.[1,2] In the past, NPC is defined as an inherited disease with varying degrees of intertumor and intratumor heterogeneity.[3] Recently, scholar explicitly advocates that the nature of NPC is an ecological disease: a multidimensional spatiotemporal "unity of ecology and evolution" pathological ecosystem, which offers an innovative theoretical framework and paradigm for our understanding of tumor complex causal processes, as well as probable preventive and therapeutic strategies for patients.[4] Nevertheless, available evidence still supports that the radiotherapy is the first choice for the treatment of NPC due to the high sensitivity of NPC cells to radiation. However, 10% of the patients develop local recurrence after radiotherapy. At present, the therapeutic strategies of locally recurrent NPC (rNPC) are mainly endoscopic surgery and re-radiotherapy.[5–7]

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The development of endoscopic surgery and a greater understanding of anatomy have allowed surgeons to conveniently treat many deep tumors without causing significant dysfunction. In addition, a technique of pedicled mucosal flap repair applied during operation can effectively reconstruct the resected wound, greatly reduce the trauma and promote recovery. A meta-analysis also demonstrated that endoscopic surgery successfully reduced surgical trauma and improved patient survival outcomes compared to open surgery.[8] Moreover, Chen et al specifically defined the endoscopic resectable area, which substantially decreased the positive margin rate to 2–7%.[9] Re-radiotherapy is one of the options for the treatment of rNPC because it can apply precision radiotherapy to the recurrent tumor sites and the rNPC is still relatively sensitive to radiation. Long-term reports have confirmed the acceptable local control rate and overall survival in patients treated with intensity-modulated radiotherapy (IMRT) for locally rNPC.[10,11] Regrettably, the incidences of reirradiation-related serious adverse effects after salvage IMRT remained significant, ranging from 34 to 75%.[10,12] Our recent studies showed that the survival rate of endoscopic surgery are superior to intensity-modulated radiotherapy.[5,13,14] Unfortunately, the 3-year overall survival (OS) of advanced patients treated with endoscopic surgery was only 59.3%,[14] and thus it is particularly critical to further improve the survival prognosis of patients with rNPC.

Hence, the scenario warrants urgent exploration of the molecular mechanisms that affect the prognosis in patients with rNPC and the identification of valuable therapeutic targets. Lgr5, a leucine-rich repeat containing G protein-coupled receptor 5, is a stem cell marker associated with several normal and cancer tissues.[15] It has been reported that the overexpression of Lgr5 is a marker of several tumors and the expression levels of Lgr5 can predict the recurrence, prognosis, and survival rates in colorectal and breast cancer.[16–18] Additionally, as a marker of cancer stem cells, Lgr5 may be used as a target for tumor therapy.[19] The present study comparatively analyzed the differential transcriptional landscape of the rNPC samples through the RNA-sequencing study and observed that the Lgr5 was also significantly up-regulated in rNPC. Consequently, we investigated the expression of Lgr5 in rNPC tissues, and analyzed its association with the clinicopathological features and disease outcomes. To the best of our knowledge, this is the first study to imply that a stem cell marker can be a promising biomarker for predicting the recurrence and prognosis in rNPC patients.

Patients and Methods

Patients and Tissue Samples

The current study acquired eleven fresh rNPC tissue specimens and five fresh adjacent normal tissue samples through biopsy for RNA sequencing. Subsequently, the 60 rNPC tissues, 30 primary NPC and 12 normal tissues were collected, in order to assess the expression of Lgr5 by means of immunohistochemistry. The primary tissues were obtained by pathological biopsy in the outpatient room. Consecutive adult rNPC patients who underwent treatment by means of salvage endoscopic nasopharyngectomy at the Department of Otorhinolaryngology of the Affiliated Eye, Ear, Nose, and Throat Hospital at Fudan University, during the time period from January 2017 to December 2018, were included in the present study. The surgical margins were negative in all the patients. The present study excluded the rNPC patients with positive surgical margins, distant metastasis, unresectable neck lymph node metastasis or tumor recurrence within six months after radiotherapy. The clinical data pertaining to the patients with rNPC, such as the pathological type, T stage, lymph node metastasis (LNM), recurrence, and survival time, were retrospectively collected from medical records. The extent and size of the tumors were determined by means of preoperative enhanced MRI examinations of the nasopharynx and the corresponding clinical stages with regard to all the rNPC patients were recorded, in accordance with the eighth edition of the American Joint Committee on Cancer Staging Manual. All the patients provided informed consent regarding the use of the clinical data. We confirmed that our study complied with the Declaration of Helsinki.

Immunohistochemical Staining of Lgr5

All the specimens were fixed in formalin, embedded in paraffin, and serial 4 μm sections were prepared to perform the immunohistochemical analysis. In short, the tissue sections were dewaxed, rehydrated, and treated with 3% hydrogen peroxide in methanol at room temperature for 15 minutes, in order to reduce the endogenous peroxidase activity. Subsequently, the slides were washed twice using deionized water and placed in phosphate-buffered saline (PBS) solution for five minutes. After incubation in 10% non-immune serum (goat) for a duration of 30 minutes, the sections

were treated with anti Lgr5 (1:400 dilution, Cambridge Abcam, UK) monoclonal antibody at 4 °C overnight. The following day, the slices were washed thrice using PBS solution. Successively, they were incubated with biotin secondary antibody for 30 minutes. Again, the cells were washed thrice using the PBS solution. Subsequently, they were incubated with streptavidin horseradish peroxidase conjugate for 20 minutes, washed thrice using the PBS solution and treated with a chromogenic agent, 0.05% diaminobenzidine. The nuclei were stained using hematoxylin. The Lgr5 expression was localized to the cell membrane.

Evaluation of Immunohistochemistry Staining

In the present study, two pathologists, who were blinded to the details regarding the patients, independently assessed the immunohistochemical expression of the antigen tested. The protein expression was evaluated using a light microscope eclipse e800 (Nikon instruments European, Amsterdam, Netherlands) under 20 times the original objective magnification. The two independent pathologists, Chen Chi and Weiyu Pan, evaluated and scored the stained slides, as per the staining intensity (negative: 0; weak: 1; medium: 2; strong: 3) and positive cell abundance (≤ 5%: 0; 6–25%: 1; 26–50%: 2; 51–75%: 3; and ≥ 76%: 4).[20,21] The final score obtained by multiplying the intensity score and the degree score was used to determine the expression of Lgr5. The scores ranging from 0–4 and 5–12 were defined as low and high expressions, respectively.

Western Blotting

The total protein was extracted with RIPA buffer, and denatured by boiling; Subsequently, the protein sample (30 ug) loaded onto 8–12% SDS/PAGE gel. After electrotransfection, the membrane was blocked with 3% BSA and incubated with primary antibody: anti-Lgr5 (Catalog No: ab273092, 1:1500). After one day of incubation with primary antibody, the membrane was incubated with anti rabbit or anti mouse IgG secondary antibody solution at room temperature at a dilution of 1:5000 (cell signal technology) for 1h, and then washed with TBST for three times for 10 minutes. The immune response band was observed by enhanced chemiluminescence Kit (abbkine, Waltham, Ma, USA).

Statistical Analysis

The chi-square test was used to analyze the relationship between the clinicopathological variables and the immunostaining of Lgr5. The Kaplan-Meier survival analysis was used to calculate the incidence of overall survival (OS) and disease-free survival (DFS) in rNPC patients and to explore the effect of Lgr5 expression on the DFS. The Log rank test was used to compare the differences between the groups. Candidate variables with a p value < 0.1 on univariate analysis were included in the multivariable model. Cox regression model was used for multivariate survival analysis. The predictive value of Lgr5 was determined by receiver operating characteristic (ROC) curve analysis. The follow-up period was defined as the period from initial surgical treatment at our institution to death or last follow-up. The value of $P<0.05$ was considered to be statistically significant. SPSS 19.0 software (IBM Corp., Armonk, NY) was used to perform the statistical analyses.

Results

Lgr5 Expression Was Up-Regulated in rNPC Tissues

The current study analyzed eleven rNPC and five normal tissue samples using the RNA-seq analysis, in order to detect the aberrantly expressed mRNAs in rNPC. The heat map revealed the 15 most up-regulated and down-regulated mRNAs (Figure 1A). In accordance with the criteria of mean fold change >2 and q-value <0.05, 204 differentially expressed mRNAs were detected, including 43 up-regulated mRNAs (Table S1) and 161 down-regulated mRNAs (Figure 1B, Table S2). The current study observed that the Lgr5 was up-regulated in rNPC and selected the same for further analysis, on account of the fact that it is a CSC marker. Moreover, the present study further validated the expression of Lgr5 in 60 rNPC, 30 primary NPC and 12 normal tissue samples using immunohistochemical staining and observed that the expression of Lgr5 was significantly higher in the rNPC tissue samples, compared to the primary NPC tissues and the normal tissues (Figure 1C and D). We also found that high Lgr5 expression was significantly correlated with the advanced T stage in rNPC (Figure 1E). Western blotting was used to detect

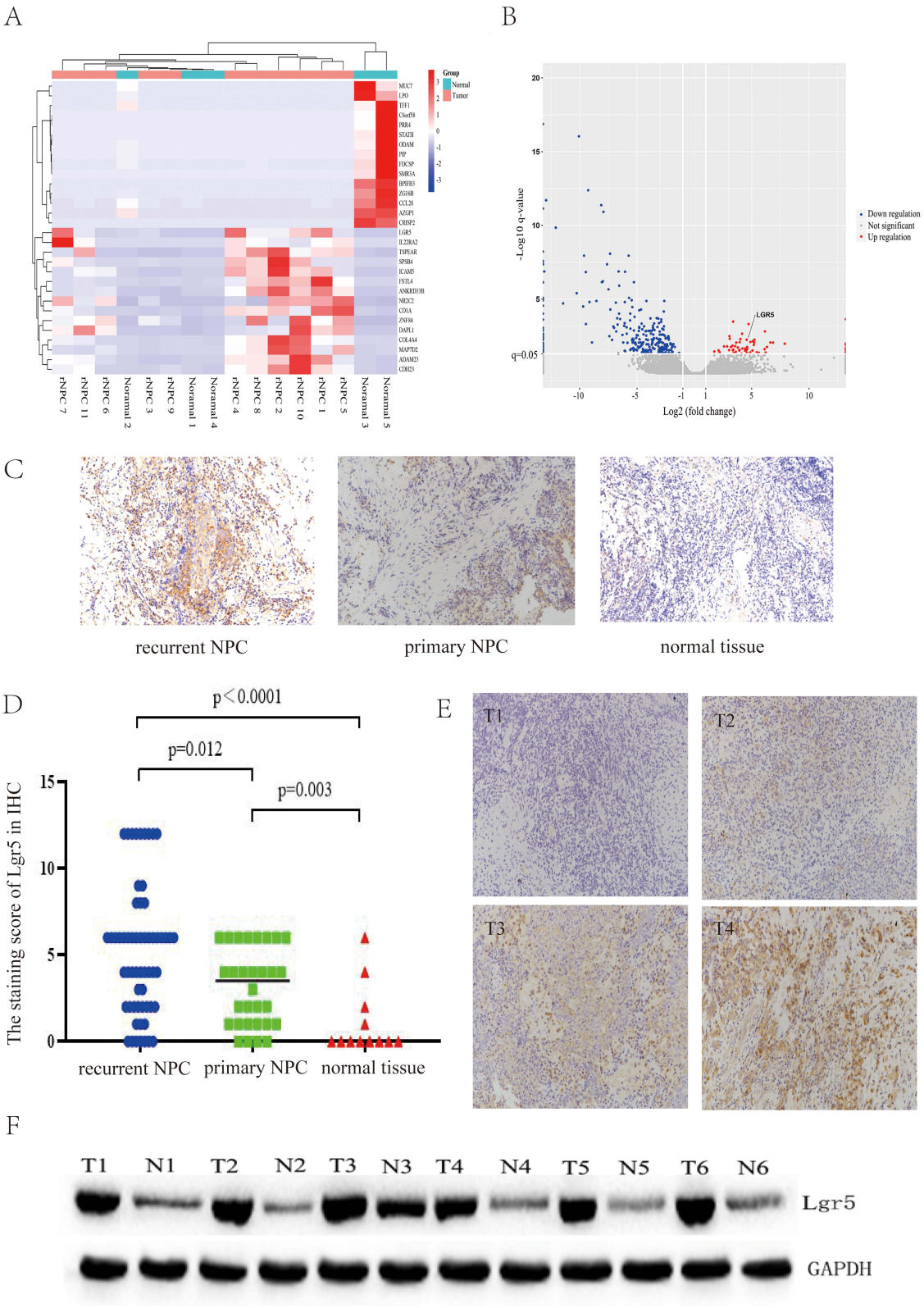

Figure 1 mRNAs expression profile in rNPC tissues compared to normal tissues. (**A**) Heat maps with hierarchical clustering analysis show 30 differentially expressed mRNAs (15 up-regulated and 15 down-regulated mRNAs) on the RNA-seq analysis. The histogram on the upper right is in color order. The color change from top to bottom indicates high-to-low mRNA expression in the sample; (**B**) Volcano plots of the differentially expressed mRNAs. Horizontal dotted line: q=0.05 (–log10 scaled); red points: upregulated mRNAs with statistical significance; green points: downregulated mRNAs with statistical significance; (**C**) Representative imaging of IHC showing the expression of Lgr5 in the rNPC, primary NPC and normal tissue samples (original magnification×200); (**D**) IHC staining score of Lgr5 was significantly higher in the rNPC tissues, compared to the primary NPC tissues and the normal tissues; (**E**) Representative imaging of IHC showing expression of Lgr5 in different T stages of rNPC. (**F**) Western blotting showed the expression of Lgr5 in 6 pairs of rNPC and non-cancer tissues.
Abbreviations: rNPC, recurrent nasopharyngeal carcinoma; IHC, immunohistochemistry.

Lgr5 protein expression in 6 pairs of rNPC and non-cancerous tissue samples; Lgr5 protein levels in rNPC tissue samples were up-regulated (Figure 1F).

Lgr5 Expression in rNPC Tissues Correlated with the Clinicopathologic Parameters

The present study identified a total of 60 patients with rNPC, among which 42 (70.0%) were male and 18 (30.0%) were female. The median age of the patients was 54 years (range: 30–75 years). A summary of the details regarding the patients is presented in Table S3. Furthermore, the correlation between the Lgr5 expression and the clinicopathological features pertaining to the rNPC patients was analyzed in terms of the significantly elevated levels of expression of Lgr5 in the rNPC tissues. The current study divided the rNPC patients into two groups, as per the level of expression of Lgr5 (low and high). The chi-square test revealed that the Lgr5 expression was significantly correlated with the T stage (P=0.014). However, there was no significant correlation with the gender, age, smoking history, drinking history, histological type, LNM, or tumor necrosis (P>0.05, Table 1).

Lgr5 Expression in rNPC Tissues Was Associated with Poor Clinical Outcomes

During the course of the median follow-up period of 31 months (2–47), the one-, two-, and three-year overall survival rates were 91.6%, 83.0%, and 76.5%, respectively (Figure 2A). The total recurrence rate of rNPC after salvage surgery was 53.3%. The one-, two-, and three-year DFS of the rNPC patients were 67.7%, 53.4%, and 39.4%, respectively (Figure 2B). The Log-rank analysis revealed lower three-year OS (66.7% vs 88.1%, P=0.007, Figure 3A) and DFS (29.6% vs 65.5%, P=0.001, Figure 4A) in the patients with high expression of Lgr5. Furthermore, tumor necrosis

Table 1 Correlation Between Lgr5 and Clinical Characteristics in 60 rNPC Patients

Variables	Lgr5 Expression Level		*P* value
	Low	High	
Gender			0.735
Male	19	23	
Female	9	9	
Age			0.916
<50	11	13	
≥50	17	19	
Smoking history			0.785
No	21	23	
Yes	7	9	
Drinking history			0.796
No	22	26	
Yes	6	6	
Histological type			0.855
WHO type II	9	11	
WHO type III	19	21	
T stage			0.014
T1+T2	21	14	
T3+T4	7	18	
LNM			0.193
No	25	24	
Yes	3	8	
Tumor necrosis			0.066
No	25	22	
Yes	3	10	

Abbreviations: rNPC, recurrent nasopharyngeal carcinoma; LNM, lymph node metastasis.

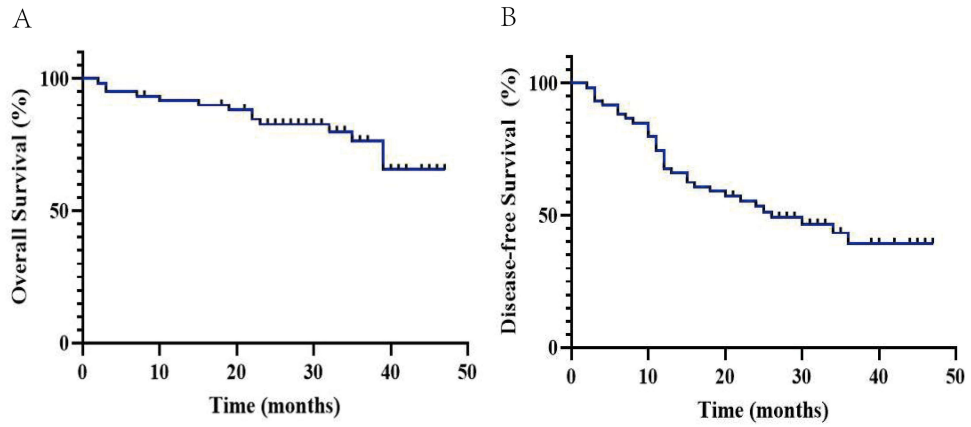

Figure 2 Kaplan-Meier curve pertaining to the survival in patients with rNPC. (**A**) Overall survival; (**B**) Disease-free survival.
Abbreviation: rNPC, recurrent nasopharyngeal carcinoma.

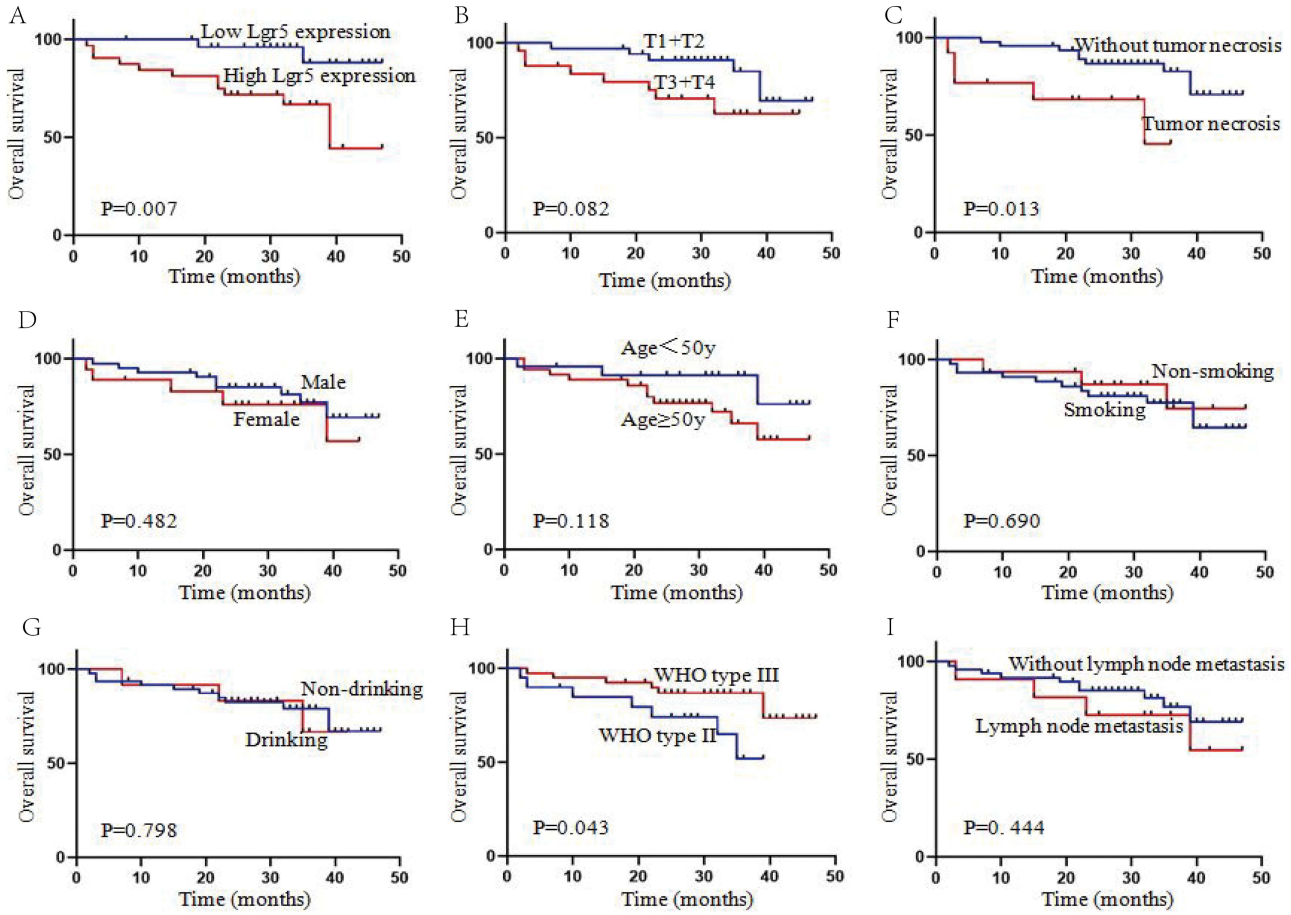

Figure 3 Kaplan-Meier curves pertaining to the OS in patients with rNPC. (**A**) Lgr5 expression (high expression vs Low expression); (**B**) T stage (T1+T2 vs T3+T4); (**C**) Tumor necrosis; (**D**) Gender; (**E**) age (<50 vs ≥50); (**F**) Smoking history; (**G**) Drinking history; (**H**) Histological type; (**I**) Lymph node metastasis.
Abbreviations: rNPC, recurrent nasopharyngeal carcinoma; OS, overall survival.

(Figure 3C, P=0.013) and WHO type II (Figure 3H, P=0.043) had a significantly worse overall survival. Similarly, advanced T stage (Figure 4B, P=0.032) and tumor necrosis (Figure 4C, P=0.012) were significantly associated with a worse disease-free survival. However, the present study did not observe any significant difference with regard to the other clinicopathological factors affecting the OS and DFS in rNPC patients (P>0.05, Table S4). The variables considered

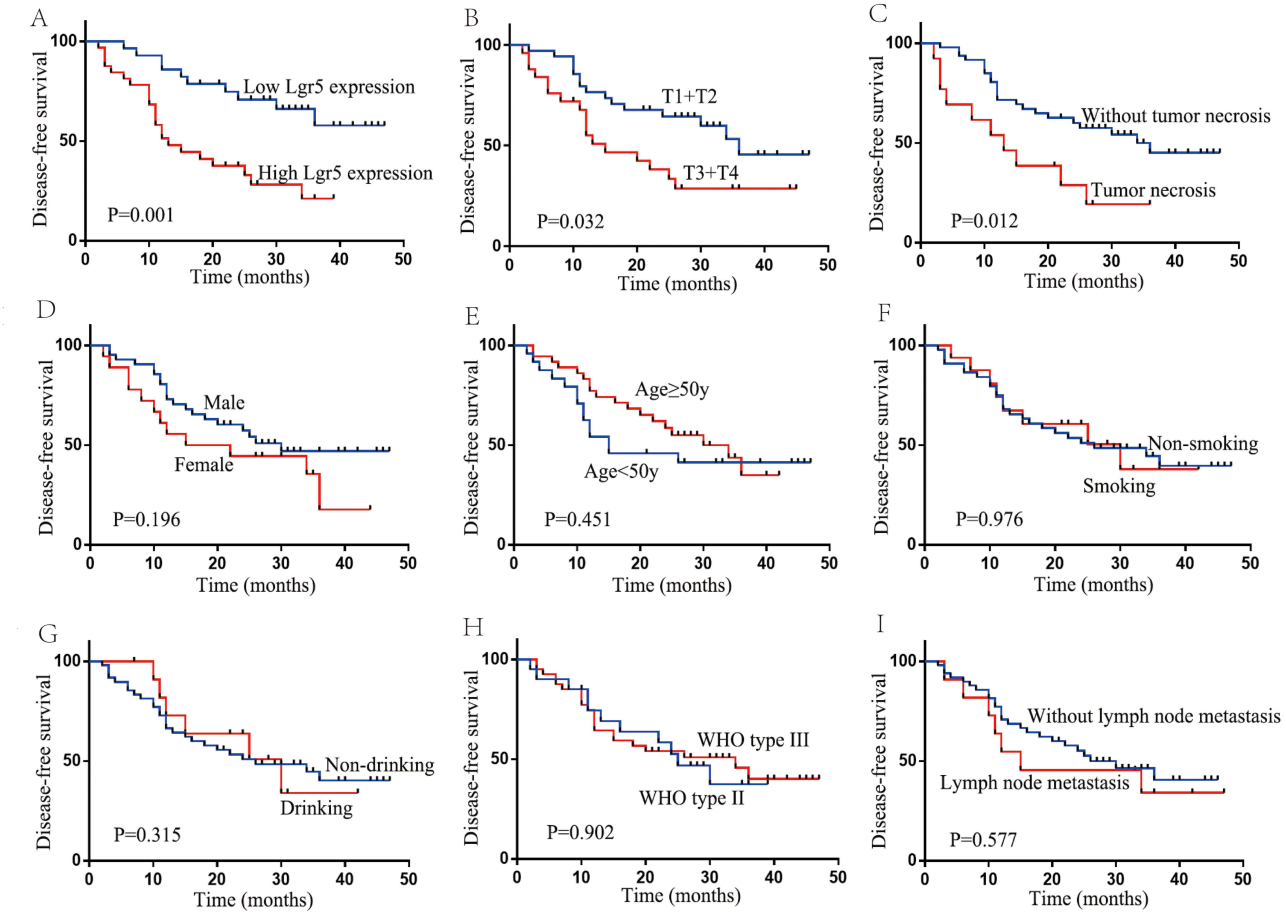

Figure 4 Kaplan-Meier curves pertaining to the DFS in patients with rNPC. (**A**) Lgr5 expression (High expression vs Low expression); (**B**) T stage (T1+T2 vs T3+T4); (**C**) Tumor necrosis; (**D**) Gender; (**E**) age (<50 vs ≥50); (**F**) Smoking history; (**G**) Drinking history; (**H**) Histological type; (**I**) Lymph node metastasis.
Abbreviations: rNPC, recurrent nasopharyngeal carcinoma; DFS, disease-free survival.

significant in Cox univariate analysis were T stage, tumor necrosis, pathological type and Lgr5 expression. Subsequently, we used the above these variables for multivariable Cox regression model, and only Lgr5 expr ession was proved to be an independent risk factor for OS and DFS (Table 2 and Table 3). Moreover, Lgr5 expression has good predictive value as a predictor of recurrence of rNPC. The area under the ROC curve for Lgr5 expression with regard to the OS was 0.711 (95% confidence interval [CI], 0.566–0.856; P=0.017, Figure 5A) and DFS was 0.665 (95% confidence interval [CI], 0.526–0.805; P=0.028, Figure 5B).

Discussion

A small population of CSCs could form larger and more tumor spheres in NPC, which was considered to be related to the radioresistance, chemoresistance, tumor recurrence, and metastasis in NPC. Conversely, previous studies have repeatedly proven that the biological functions of several signaling pathways associated with cancer possess the characteristics of stemness and the potential of self-renewal.[22,23] Several distinctive biological characteristics make these cells outstanding targets for biomarker identification. Consequently, a large number of CSC markers have been identified, such as the CD133, CD44, and ALDH1.[24–26] It has been suggested that resistant CSCs are the main cause of the treatment failure in NPC.[22,27] On the basis of the aforementioned background, specific biomarkers may be the key to the elimination of NPC CSCs and the biomedical technology and treatment protocols that target the NPC CSCs are the inevitable determinants of the therapy.

Lgr5, also known as G protein-coupled receptor 49 (GPR49), is a Wnt signaling pathway target gene that activates R-sponge protein 1 (RSPO1) and Wnt-3a, and cooperates with the Frizzled-5 (Fzd5) and LDL receptor related protein 6

Table 2 Univariate and Multivariate Cox Regression Analyses of OS in Patients with rNPC

Variable	Univariate			Multivariate		
	HR	95% CI	P	HR	95% CI	P
The expression of Lgr5						
Low expression	1		0.019	1		0.035
High expression	6.047	1.351–27.074		5.435	1.130–26.147	
T stage						
T1+T2	1		0.095	1		0.997
T3+T4	2.491	0.854–7.266		1.002	0.248–4.052	
Tumor necrosis						
No	1		0.021	1		0.304
Yes	3.911	1.226–12.478		1.967	0.542–7.136	
Histological type						
WHO type II	1		0.054	1		0.129
WHO type III	0.344	0.116–1.019		0.370	0.102–1.336	
Gender						
Male	1		0.488			
Female	1.473	0.493–4.402				
Age						
<50	1		0.136			
≥50	2.644	0.737–9.49				
Smoking history						
No	1		0.693			
Yes	0.772	0.214–2.787				
Drinking history						
No	1		0.799			
Yes	1.182	0.326–4.288				
LNM						
No	1		0.452			
Yes	1.564	0.488–5.014				

Abbreviations: rNPC, recurrent nasopharyngeal carcinoma; OS, overall survival; LNM, lymph node metastasis.

Table 3 Univariate and Multivariate Cox Regression Analyses of DFS in Patients with rNPC

Variable	Univariate			Multivariate		
	HR	95% CI	P	HR	95% CI	P
The expression of Lgr5						
Low expression	1		0.002	1		0.018
High expression	3.279	1.536–7.002		2.711	1.188–6.188	
T stage						
T1+T2	1		0.039	1		0.690
T3+T4	2.087	1.040–4.189		1.186	0.513–2.744	
Tumor necrosis						
No	1		0.016	1		0.281
Yes	2.518	1.184–5.355		1.618	0.674–3.881	
Gender						
Male	1		0.205			
Female	1.591	0.776–3.260				
Age						
<50	1		0.458			
≥50	0.767	0.380–1.546				

(*Continued*)

Table 3 (Continued).

Variable	Univariate			Multivariate		
	HR	95% CI	P	HR	95% CI	P
Smoking history						
No	1		0.977			
Yes	0.988	0.443–2.203				
Drinking history						
No	1		0.860			
Yes	0.923	0.379–2.249				
Histological type						
WHO type II	1		0.903			
WHO type III	0.956	0.460–1.984				
LNM						
No	1		0.582			
Yes	1.267	0.546–2.939				

Abbreviations: rNPC, recurrent nasopharyngeal carcinoma; DFS, disease free survival; LNM, lymph node metastasis.

(LRP6).[28] It is expressed in many tissues and organs as a marker for homeostatic stem cells, including the hair follicles[29] and antral stomach.[30] Lgr5 plays an important role in the embryogenesis, tumor development, and signal transduction in tumor cells.[15] Certain recent reports have shown that Lgr5 is highly expressed in a variety of malignant tumors, enhances the Wnt/β-catenin signaling pathway, and stimulates the proliferation and self-renewal of CSCs in tumors such as the colorectal tumors[31] and Ewing sarcoma.[32] Similarly, a previous study by the authors observed that the Lgr5 marked the stem cells and regulated the stemness, migration, invasion, and drug resistance via the Wnt/β-catenin signaling in primary NPC cells.[33] However, the role of Lgr5 in the prognosis of rNPC patients remains ambiguous.

The present study used the microarray analyses and found that the Lgr5 was up-regulated in the rNPC tissue samples, compared to the normal tissue samples. Similarly, the immunohistochemical and Western blotting analysis revealed that the expression of Lgr5 in rNPC tissues was significantly higher, compared to the normal tissues. Moreover, our data

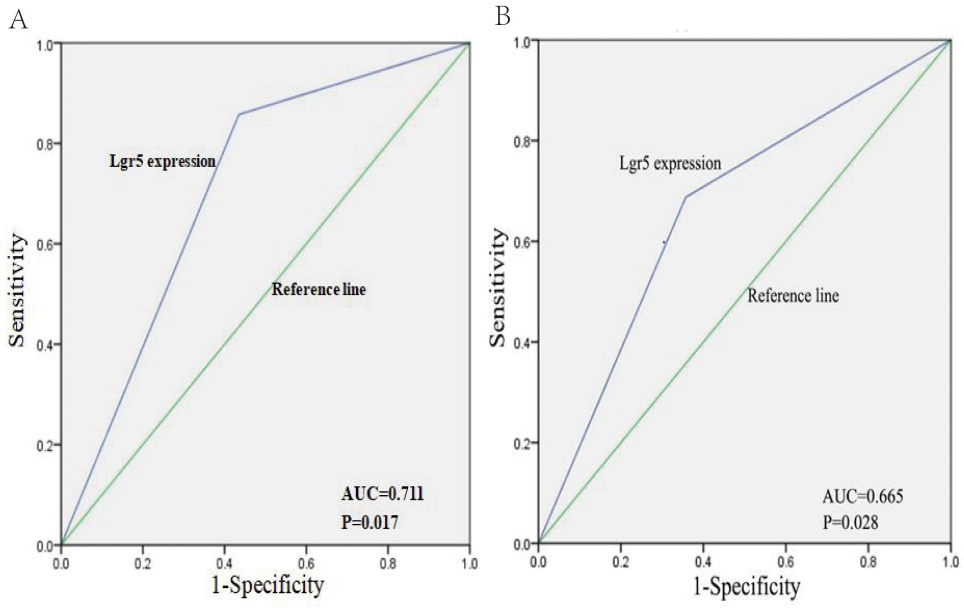

Figure 5 Receiver operating characteristic analysis revealed that Lgr5 expression had good predictive value as a predictor of survival and recurrence in rNPC patients. The area under the ROC curve was 0.711 (**A**) (P=0.017) and 0.665 (**B**) (P=0.028), respectively.
Abbreviations: rNPC, recurrent nasopharyngeal carcinoma; ROC, receiver operating characteristic.

suggested that elevated Lgr5 expression was strongly associated with the advanced T stage in rNPC. Our previous study also confirmed that high levels of Lgr5 expression were significantly associated with the advanced T stage in NPC patients.[33] Similarly, high expression of Lgr5 was significantly associated with poor T stage in gastric cancer and colorectal cancer.[34,35] In addition, the multivariable Cox regression revealed that the high Lgr5 expression in rNPC patients was significantly associated with worse OS and DFS. Hence, the aforementioned findings indicate that the Lgr5 is responsible for the poor prognosis in patients with rNPC.

The current study has certain limitations and it is important to address the same. First, the present study only collected the samples of rNPC after salvage surgery during the time period from 2017 to 2018, hoping to collect more samples and conduct a longer follow-up to confirm the relationship between Lgr5 expression and the clinical outcomes in rNPC. In addition, $Lgr5^{+}$ and $Lgr5^{-}$ cells should be isolated from the rNPC tissues and further functional studies should be carried out, including the research involving the proliferation, cell cycle, radioresistance, and chemoresistance, which may ultimately confirm the potential rNPC markers for CSCs. Finally, research should continue to explore the underlying mechanisms associated with the effect of Lgr5 on the biological behavior of rNPC through the Wnt pathway, which is of great importance, owing to its clinical significance and the targeted therapy for rNPC.

Conclusions

The present study confirmed the high expression of Lgr5 in rNPC and its important clinicopathological significance, suggesting that Lgr5 not only plays an important role in the occurrence and development of rNPC, but also serves as a candidate marker that can be used as a new prognostic indicator in rNPC. Hence, Lgr5 is a promising target that could be employed as an effective therapeutic strategy to control the rNPC on the basis of the CSC markers.

Data Sharing Statement

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Ethics Approval

The current study was approved by the Institutional Review Board of the Affiliated Eye, Ear, Nose, and Throat Hospital at Fudan University (Approved number: 2021079).

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Disclosure

The authors declare that they have no competing interests.

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