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RESEARCH ARTICLE

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RFC4 promotes the progression and growth of Oral Tongue squamous cell carcinoma in vivo and vitro

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Abstract

Objective: Currently, many studies have found that RFC4 was up-regulated in various cancers, and related to the progression and development. While the effects of RFC4 in oral tongue squamous cell carcinoma remain unclear, the main purpose of this research is to explore the role of RFC4 in oral tongue squamous cell carcinoma.

Methods: The expression of RFC4 in various cancers was analyzed in GEPIA database, and the results were further verified by IHC assay. The relationship between RFC4 and several clinical parameters was analyzed; the proliferation was further observed by knockdown RFC4 in vitro. Finally, we constructed related nude mouse models by planting cells subcutaneous of nude mice, and the discrepancy was observed.

Results: Based on GEPIA database, RFC4 was up-regulated in various cancers, including colorectal cancer, breast cancer, prostate cancer, lung cancer, and liver cancer. RFC4 was up-regulated in oral tongue squamous cell carcinoma compared with the normal tissue from GEPIA online database; we further found that the expression of RFC4 was tightly associated with TNM stage ($p = 0.005$), but not with age, gender, and differentiation ($p > 0.05$). We further found that the proliferation of oral tongue squamous cell carcinoma was obviously restrained in vitro, and the carcinogenesis was also inhibited in vivo.

Conclusions: We found that RFC4 was up-regulated and related to the progression of oral tongue squamous cell carcinoma, and knockdown RFC4 could restrain the proliferation and progression. RFC4 might serve a potential biomarker and provide a new treatment strategy for lots of patients with oral tongue squamous cell carcinoma.

KEYWORDS
oral tongue squamous cell carcinoma, progression, proliferation, RFC4

1 | INTRODUCTION

Currently, oral tongue squamous cell carcinoma has been the one of the most common oral cavity and pharynx tumors all over the world. Based on the cancer statistics in 2020 from America,[1] cardiovascular diseases were still the most common reason causing death among the world, while cancers were the second common reason and they are increasing daily. According to the statistical analysis, there were almost 17660 new cases with oral tongue squamous cell carcinoma in 2020, of which the percentage of male patients was more than twice compared with the percentage of female patients. And there were approximately 2830 deaths derived from oral tongue squamous cell carcinoma. And the surgical excision was

Jian Zhang and Linlin Wang contributed equally to this article.

still the mainstream treatment for lots of patients.[2] However, whether elective neck dissection (END) should be included in the treatment of early-stage lesions (cT1-2N0) of the tongue remained controversial.[3] There were many factors caused the occurrence and progression of oral tongue squamous cell carcinoma[4]; many patients lost the timing of treatment in that they were diagnosed as late stage.[5] So, it was becoming more and more urgent for us to look for a sensitive biomarker and treat target for lots of patients with oral tongue squamous cell carcinoma.[6]

RFC4 (Replication Factor C Subunit 4) is a protein complex consisting of five distinct subunits of 140, 40, 38, 37, and 36 kD. This gene encodes the 37 kD subunit. This subunit forms a core complex with the 36 and 40 kDa subunits. The core complex possesses DNA-dependent ATPase activity, which was found to be stimulated by PCNA in an in vitro system. And RFC4 was required for the elongation of primed DNA templates by DNA polymerase delta and DNA polymerase epsilon.[7] The dysregulation of RFC4 was associated with many diseases, such as Homologous DNA Pairing and Strand Exchange and Gastric Cancer, the role in the diagnosis and progression of various cancers. For example, Yunan He et al identified RFC4 as the critical prognostic biomarkers in cervical squamous carcinoma via bioinformatic data.[8] And Maria Gisatulin et al found that the RFC4 family member RFC1 was tightly related to ataxia syndromes.[9] Arai M et al reported that RFC4 was closely related to the prognosis of liver cancer.[10] All above researches implied that RFC4 was an essential factor, and it was associated with the progression of various cancers. However, the role of RFC4 in oral tongue squamous cell carcinoma remains unclear, the main purpose of this research is to preliminary explore the relationship between RFC4 and oral tongue squamous cell carcinoma. RFC4 might provide a new treatment strategy for lots of patients with oral tongue squamous cell carcinoma.

2 | METHODS

2.1 | Patient information

These patients with oral tongue squamous cell carcinoma involved in this research were informed and consent. And these tissue of all 64 patients with oral tongue squamous cell carcinoma in this research derived from our hospital. The processes of collecting tissue were performed by professional doctor at an aseptic environment.

2.2 | Reagents and antibodies

Western blotting-related antibodies: anti-RFC4 (ab156780) Rabbit 1:1000; Anti-GAPDH (ab8245) Mouse 1:500. IHC assay related antibodies: anti-RFC4 (ab156780) Rabbit 1:150.

2.3 | Cells and transfection

Oral tongue squamous cell carcinoma CAL27 and TCA8113 cells were purchased from ATCC, and CAL27 was cultured with DMEM containing 10%FBS and 1% penicillin-streptomycin double antibody, and TCA8113 was cultured with 1640 containing 10% FBS and 1% penicillin-streptomycin double antibody in 37℃ and 5%CO_2. And based on the transfection protocol, CAL27 and TCA8113 cells were transfected with RFC4 shRNA for subsequent experiments. Pre-designed four RFC4 shRNA sequences were as follows: shRNA 1: AAGGATCGAGGAGTAGCTGCCAG; shRNA 2: GGACCACCTGGAACTGGAAAAAC; shRNA 3: CCGTGTCC GCCTTTTAAGATTGT; shRNA 4: AAAATTCCGCTTCAAGCCTCTGT.

2.4 | Data extraction

The related expression of RFC4 in oral tongue squamous cell carcinoma and normal tissue was extracted from GEPIA online database (https://www.proteinatlas.org/ENSG00000163918-RFC4/tissue).

2.5 | RNA extracting and qRT-PCR

We extracted the total RNA from CAL27 and TCA8113 cells transfected with RFC4 shRNA according to Trizol assay protocol for subsequent PCR-related assays. And the primers of GAPDH: 5'-CGACCACTTTGTCAAGCTCA-3' and 5'-GGTTGAGCACAGGGT ACTTTATT-3'.

2.6 | Western blotting

The total protein was extracted from CAL27 and TCA8113 cells transfected with RFC4 shRNA according to RIPA assay, and protein was used for subsequent western blotting. The change of the expression of RFC4 was further observed.

2.7 | Immunohistochemical staining

Based on the procession of the immunohistochemical staining kit, these paraffin specimens were sliced at 3–4 μm and grilled slices at 65℃ for 50 min, and soaking in following order, xylene solution (1) for 10 min, xylene solution (2) for 10 min, absolute ethanol solution (1) for 5 min, absolute ethanol solution (2) for 5 min, 95% alcohol solution for 5 min, 85% alcohol solution for 5 min, and 75% alcohol solution for 5 min. Washed in PBS buffer for 2 minutes two times. And placed the slices in sodium citrate buffer in the microwave (high fire for 5 min firstly and then low to medium fire for 7–10 min). Then, placed at room temperature for 2 h and wash with PBS buffer for 2 min two times. Removed excess water and drop H2O2 in the wet box for 20 min at room temperature, washed again, and dropped BUB3 and CDCA3 antibodies for 4℃ overnight. In next day, placed at room temperature for one hour, washed with PBS buffer for 2 min two times, dropped IgG secondary antibodies for 2 h at room temperature. And wash again with PBS buffer,

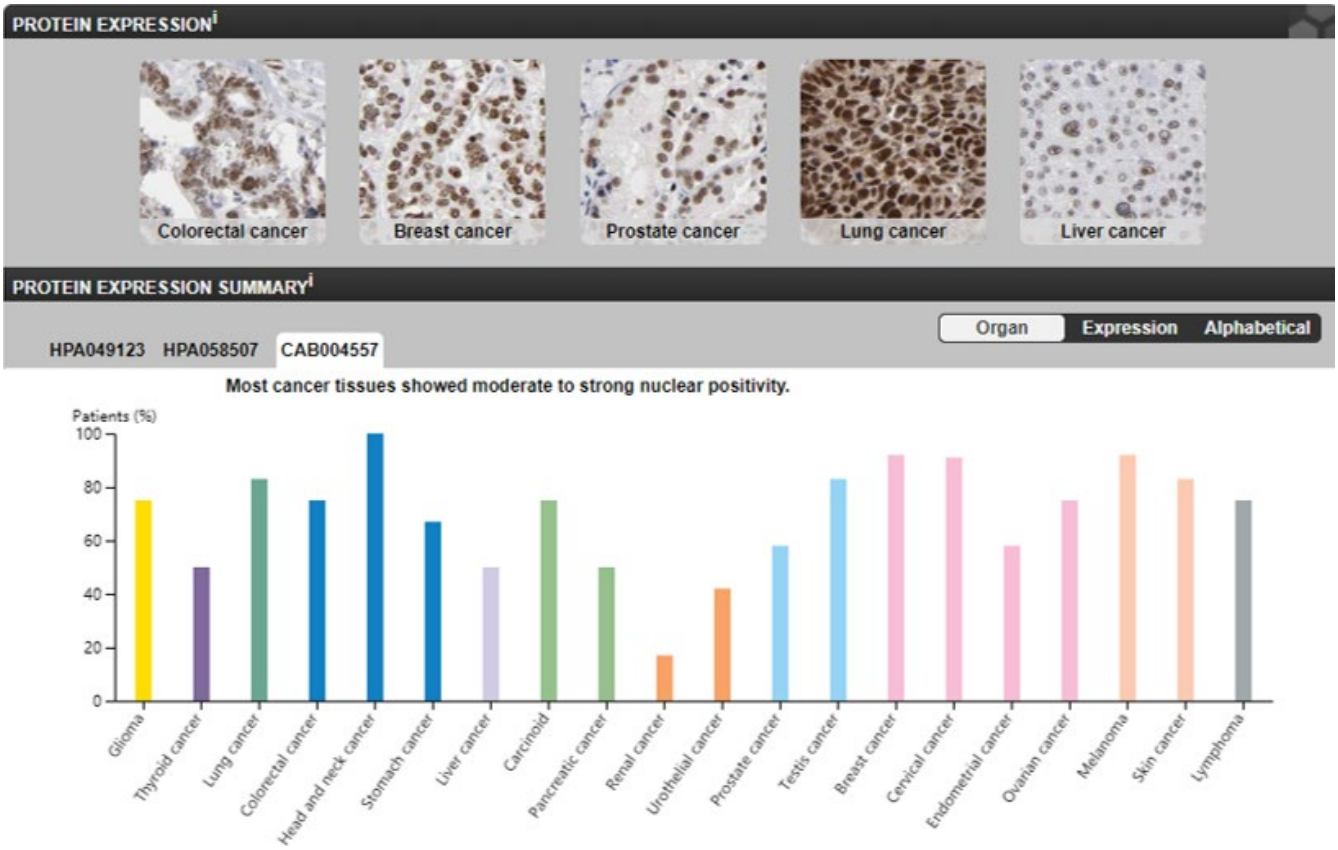

FIGURE 1 RFC4 was up-regulated in various cancers. The expression of RFC4 in several cancers, including colorectal cancer, breast cancer, prostate cancer, lung cancer, and liver cancer

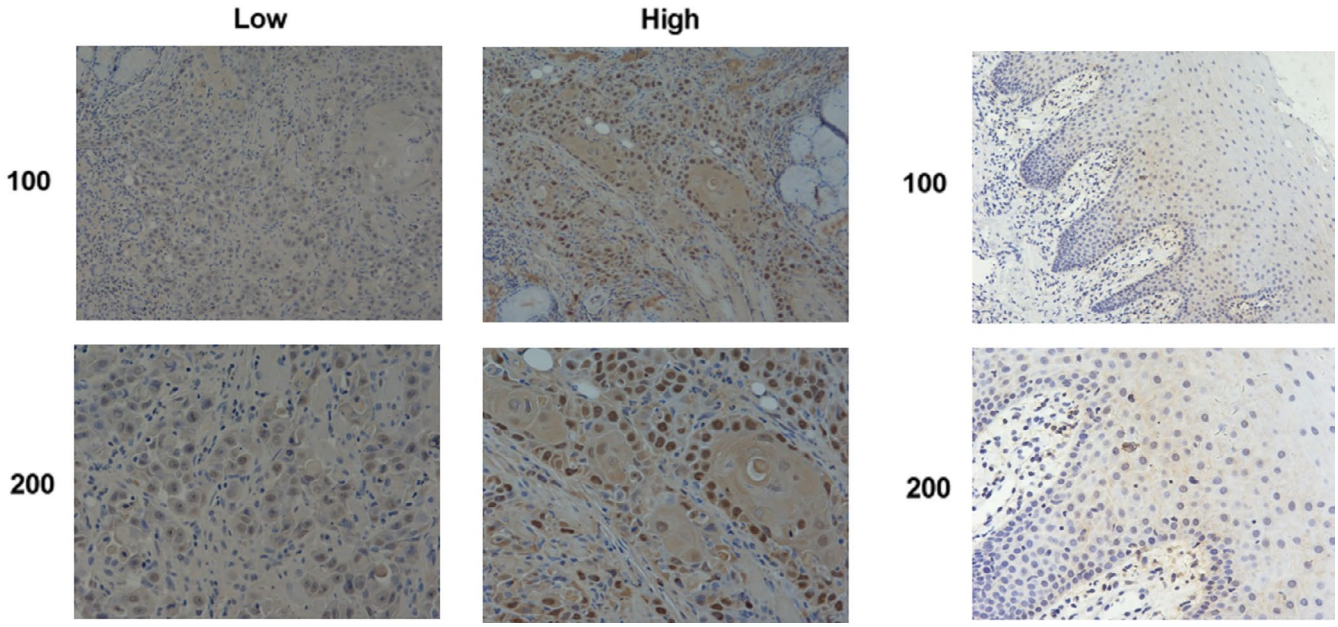

FIGURE 2 The expression of RFC4 in oral tongue squamous cell carcinoma. Left panel: The expression of RFC4 in oral tongue squamous cell carcinoma (IHC), tissues were classified as low expression and high expression. Right panel: The expression of RFC4in normal tissues was used as normal control group. The expression of rfc4 in normal tissue is low and stain is weakly or negative

drop DAB (pre-formulated with liquid A and liquid B), staining at room temperature about 5–15 s. Stop dyeing with water and next stain nucleus with hematoxylin solution for 5–8 s, tap water rinse for 2 min. Soaking in following order, 75% alcohol solution, 85% alcohol solution, and 95% alcohol solution each for 1 min, absolute ethanol (1) and absolute ethanol (2) each for 3 min, xylene (1) and xylene (2) each for 5 min. Finally, neutral gum sealer and look under the microscope.

2.8 | Colony-forming assays

CAL27 and TCA8113 cells transfected with RFC4 shRNA were counted and placed 2000 into each well of 6-well plate. And cells were cultured for additional 5–6 days, fix with 4% paraformaldehyde, and stain with crystal violet.

2.9 | CCK-8 assay

CAL27 and TCA8113 cells transfected with RFC4 shRNA with 3000 per well were planted into 96-well plate; these cells were cultured for additional 72 h. The remain 1640 culture was extracted, and 10ul CCK-8 reagent was added, incubated in 37℃, and detected 570 nm absorbance.

2.10 | Animal assay

CAL27 and TCA8113 cells transfected with RFC4 shRNA were planted to subcutaneous of nude mice; the volume of ectopic tumors was observed daily for 25 days. And these tumors were extracted and embedded with paraffin for subsequence immunohistochemical staining to detect the expression of RFC4.

2.11 | Statistical analysis

Software Graphpad prism 8 was used to analyze the results data, and we used t test and chi-square analysis to analyze the role of RFC4 in oral tongue squamous cell carcinoma.

3 | RESULTS

3.1 | RFC4 was up-regulated in various cancers

It has been known that RFC4 was related to the progression of various cancers, and we further analyzed the expression of RFC4 in several cancers from GEPIA online database. We found that RFC4 displayed moderate to strong nuclear positivity. Based on the database, we filtered several cancers and detected the expression levels of RFC4, containing colorectal cancer, breast cancer, prostate cancer, lung cancer, and liver cancer, and the results demonstrated that RFC4 showed strong staining in these cancers, especially in lung cancer (Figure 1). And we further found that RFC4 displayed high expression, but the expression of RFC4 was low in renal cancer (Figure 1). All above results showed that RFC4 was up-regulated in various cancers, and which might be associated with the progression and development of cancers.

3.2 | RFC4 was up-related in oral tongue squamous cell carcinoma and associated with TNM stage

To further explore the role of RFC4 in oral tongue squamous cell carcinoma, we collected 64 paired samples with oral tongue squamous cell carcinoma and performed the immunohistochemical staining assay to detect the expression of RFC4 in oral tongue squamous cell carcinoma and the normal tissue. The results demonstrated that the expression of RFC4 was significantly high in oral tongue squamous cell carcinoma compared with the normal tissue (Figure 2). The results were consistent with the results of database; all above results implied that the expression of RFC4 might be associated with the occurrence and development of oral tongue squamous cell carcinoma. So, we divided these 64 patients into high and low groups based on the staining intensity of RFC4. Then, we further explore the relationship between the expression of RFC4 and several clinicopathological parameters (including age, gender, differentiation, and TNM stage) of oral tongue squamous cell carcinoma (Table 1). The results demonstrated that the expression of RFC4 was related to TNM stage ($p = 0.005$), but not with age, gender, and differentiation ($p > 0.05$). In summary, we have reason to believe that RFC4 is highly expressed in oral tongue squamous cell carcinoma and plays an important role in the clinical progress of oral tongue squamous cell carcinoma.

TABLE 1 Relationships of RFC4 and clinicopathological characteristics in 64 patients with tongue squamous cell carcinoma

		RFC4 expression			
		Low	High		
Feature	All n = 64	n = 28	n = 36	χ^2	p
Age (year)					
<60	34	14	20	0.195	0.659
≥60	30	14	16		
Gender					
Male	36	17	19	0.403	0.525
Female	28	11	17		
Differentiation					
Low	30	16	14	2.107	0.147
High	34	12	22		
pTNM stage					
I-II	33	20	13	7.866	0.005
III-IV	31	8	23		

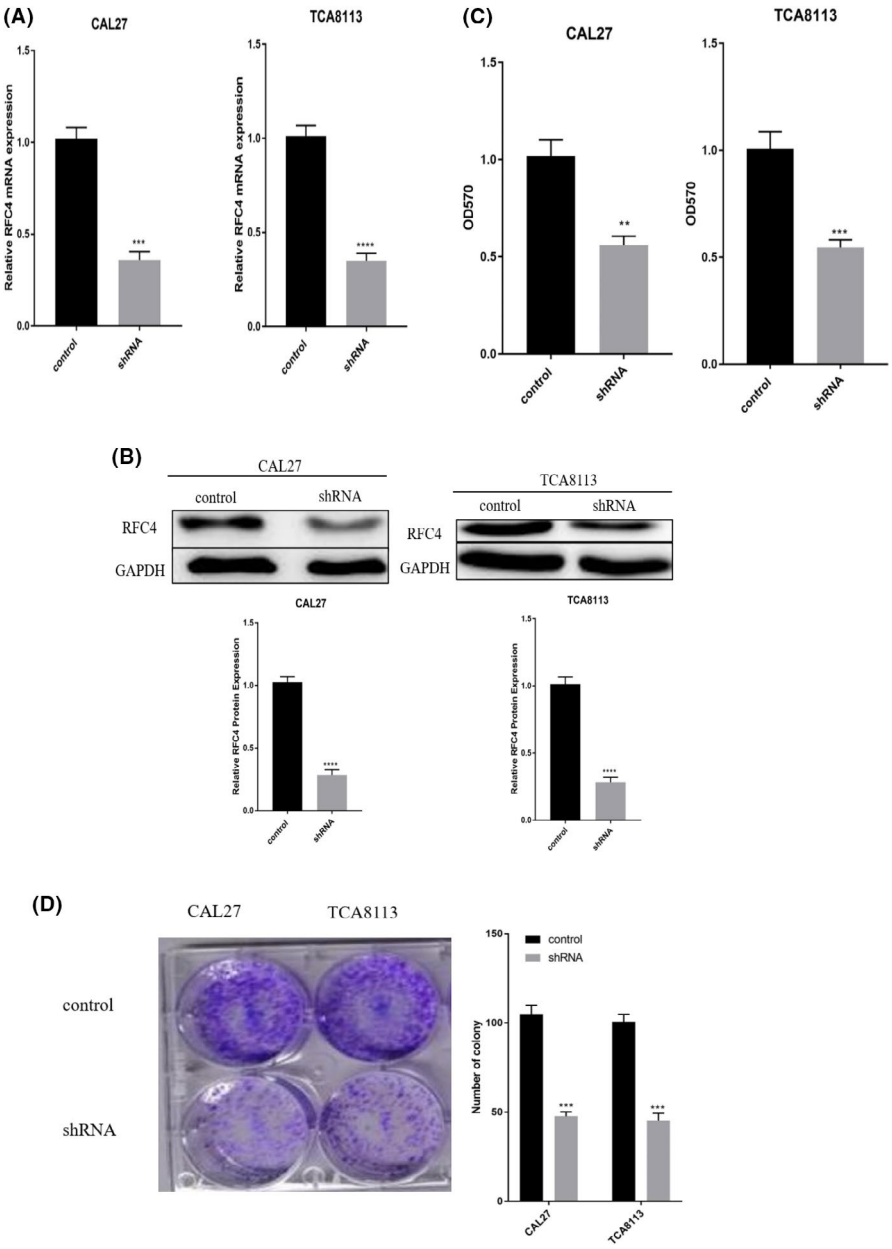

FIGURE 3 Knockdown RFC4 could restrain the proliferation of oral tongue squamous cell carcinoma CAL27 and TCA8113 cells. A. The mRNA expression of RFC4 in CAL27 and TCA8113 cells transfected with RFC4 shRNA. B. The protein expression of RFC4 in CAL27 and TCA8113 cells transfected with RFC4 shRNA. C. The CCK-8 assay of CAL27 and TCA8113 cells transfected with RFC4 shRNA. D. The colony-forming assay of CAL27 and TCA8113 cells transfected with RFC4 shRNA

3.3 | Knockdown RFC4 restrained the proliferation of oral tongue squamous cell carcinoma in vitro

To further identify the role of RFC4 in the progression and proliferation of oral tongue squamous cell carcinoma, we knockdown the expression of RFC4 via transfecting oral tongue squamous cell carcinoma CAL27 and TCA8113 cells with RFC4 shRNA. Firstly, we extracted the total RNA from CAL27 and TCA8113 cells transfected with RFC4 shRNA and relative PCR assay was conducted to detect the change; the results showed that the mRNA expression of RFC4 was restrained obviously compared with the control group (Figure 3A). Next, we continued to extract protein for western blotting to detect the protein expression of RFC4, and we found that the protein levels of RFC4 were declined significantly in RFC4 knockdown group than the control group (Figure 3B). To further explore the effects of RFC4 on the proliferation and progression of oral tongue squamous cell carcinoma in vitro, we performed CCK-8 assay and colony-forming assay to explore the proliferation ability. The results of CCK-8 assay showed that the proliferation ability of CAL27 and TCA8113 cells was significantly restrained in RFC4 knockdown group compared with the control group (Figure 3C). We further verified the conclusion via colony-forming assay; the results showed that the colony-forming ability was also inhibited obviously in RFC4 knockdown group than the control group (Figure 3D). All above results demonstrated that RFC4 promoted the proliferation of oral tongue squamous cell carcinoma and was associated with the progression of oral tongue squamous cell carcinoma in vitro. RFC4 might provide a new treatment strategy for lots of patients with oral tongue squamous cell carcinoma.

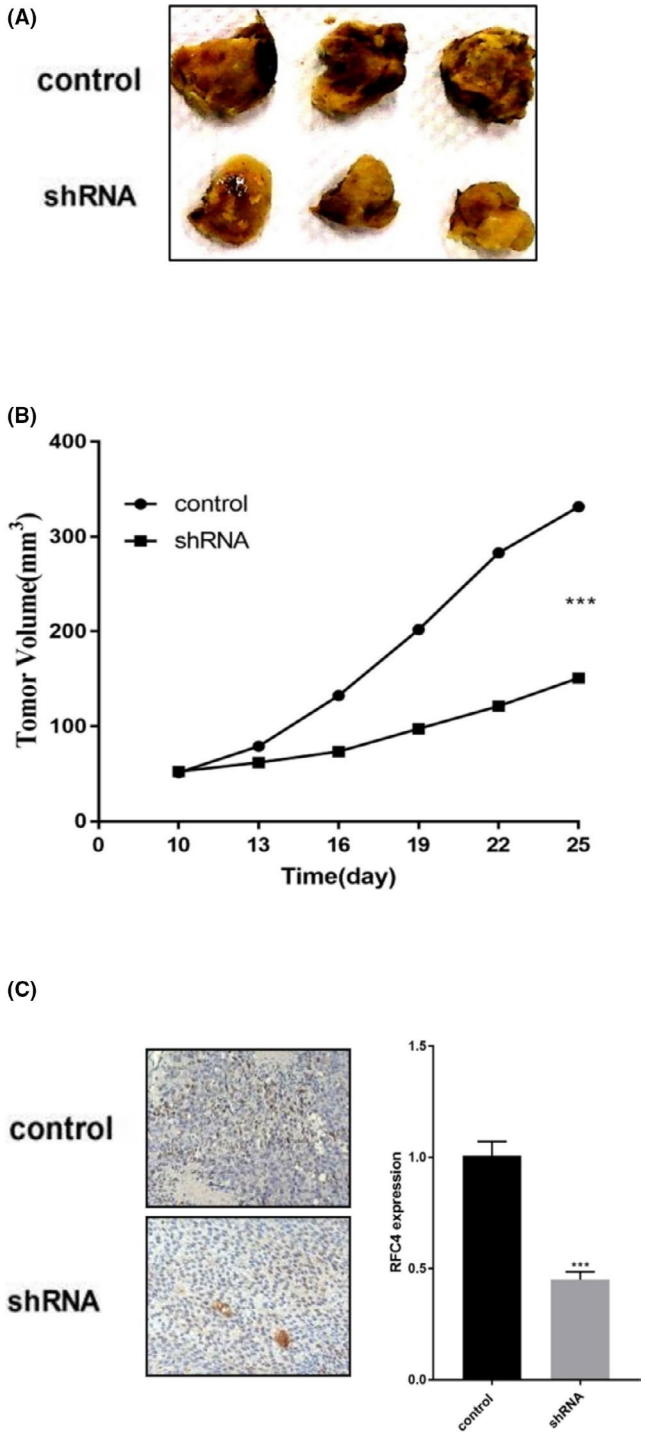

FIGURE 4 Knockdown RFC4 inhibited the growth of oral tongue squamous cell carcinoma in vivo. A, B. CAL27 cell transfected with RFC4 shRNA was transplanted subcutaneously in nude mice; the tumor volume was measured with a caliper. C. IHC staining with RFC4 was performed in these tumors from nude mice

3.4 | Knockdown RFC4 inhibited the growth of oral tongue squamous cell carcinoma in vivo

To further consolidate the above results, we planted CAL27 and TCA8113 cells transfected with control plasmid and RFC4 shRNA to the subcutaneous of nude mice to observe the effects of RFC4 on the growth of oral tongue squamous cell carcinoma in vivo. We began to measure the volume of tumors daily when the volume reached to 50 mm^3. After 25 days of planting cells, we removed these tumors from mice, and we found that the volume of tumors of CAL27 and TCA8113 cells transfected with RFC4 shRNA was restrained obviously compared with the control group (Figure 4A). And the growth curve of tumors showed that the growth rate of these tumors with RFC4 shRNA group was significantly inhibited than the control group (Figure 4B). To further verify the effects of RFC4 on the growth, we embedded these tumors and performed immunohistochemical staining assay to observe the expression of RFC4. The results showed that the expression of RFC4 was obviously low in RFC4 shRNA group compared with the control group (Figure 4C). All above results demonstrated that RFC4 promoted the growth of CAL27 and TCA8113 cells in vivo. So far, we have enough reasons to believe that RFC4 promotes the proliferation and growth of oral tongue squamous cell carcinoma in vitro and vivo. And the internal mechanism of RFC4 promoting the progression of oral tongue squamous cell carcinoma needs the rest research. RFC4 might provide a new treatment strategy for lots of patients with oral tongue squamous cell carcinoma.

4 | DISCUSSIONS

Oral tongue squamous cell carcinoma was still one of the malignant tumors threatening human life among oral cancers because of its high mortality rate.[11] And based on the statistical analysis, oral tongue squamous cell carcinoma approximately accounted for one third of all cancers of oral.[1] Although the percentage of oral tongue squamous cell carcinoma was low among all cancers, the new cases every year were should not be ignored,[12] and looking for a sensitive diagnosis-related biomarker and potential treatment was the most urgent thing currently for lots of patients.[13] RFC4 has been known to be involved in the elongation of the multi-primed DNA template, and the role of RFC4 implied that RFC4 might regulate the DNA duplication and proliferation.[14] Previous researches have proved that RFC4 was up-regulated in various and associated with the progression[15]; to further verify the results, we extracted information from online database and found that RFC4 was high expression in various cancers, especially in lung cancer. These results were consistent with the conclusion of many scholars. We further analyzed the expression of RFC4 in oral tongue squamous cell carcinoma by collecting 64 paired samples with oral tongue squamous cell carcinoma and performing immunochemistry; the results showed that the expression of RFC4 was up-regulated in oral tongue squamous cell carcinoma compared with the normal tissue. To explore the relationship between RFC4 and the clinical pathology of oral tongue squamous cell carcinoma, we divided these patients into high and low groups based on the staining intensity of RFC4, and we found that RFC4 was associated with TNM stage. Interesting that, LaTulippe E et al also identified that RFC4

was up-regulated in prostate cancer and related to the metastasis.[16] And Erdogan E et al also found that RFC4 was tightly associated with the progression of lung cancer.[17] Jung HM et al identified that RFC4 was tightly related to the progression of leukemia.[18] The role of RFC4 as a promoting cancer factor has been identified in various cancers. To explore the possibility of targeting RFC4 for oral tongue squamous cell carcinoma, we knockdown RFC4 and observed the change of proliferation and growth in vitro and vivo. The results showed that RFC4 promoted the proliferation of oral tongue squamous cell carcinoma in vitro and promoted the growth rate of oral tongue squamous cell carcinoma in vivo. Szymanska Z et al identified that RFC4 was highly expressed throughout the cell circle process of proliferating cells, and tumor proliferation in situ will become slow with the development of the disease,[19] which might be the reason why RFC4 would promote the proliferation of oral tongue squamous cell carcinoma. Fatima A et al identified that RFC4 was an independent predictor of overall survival in breast cancer.[20] And the conclusion of Niu G et al was consistent with the previous research.[21] We have enough reasons to predict that RFC4 was also an independent predictor for oral tongue squamous cell carcinoma, and targeting RFC4 was a potential treatment strategy for lots of patients with oral tongue squamous cell carcinoma.

In summary, we preliminarily determine that RFC4 promotes the proliferation in vitro and growth in vivo, and RFC4 might be used as a diagnosis-related biomarker and a potential treatment strategy for oral tongue squamous cell carcinoma. However, this conclusion needs further experiments to strengthen in that this research is a small samples and single-center study. We need to storage more patient samples and conduct a multicenter and complete study, and we next will focus on digging the deeper internal relationship and mechanism between RFC4 and oral tongue squamous cell carcinoma.

5 | CONSENT OF PUBCALITION

Informed consent of all authors.

CONFLICTS OF INTEREST

None of the authors have any relevant conflicts of interest pertaining to the studies and data in this manuscript.

AUTHOR'S CONTRIBUTION

Jian Zhang involved in projective development and manuscript writing. Linlin Wang involved in manuscript writing and performing experiments. Xiao Xie involved in extracted database and performing experiments.

DATA AVAILABILITY STATEMENT

All data and material are Availability.

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