CircRFWD3 promotes HNSCC metastasis by modulating miR-27a/b/PPARγ signaling

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INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC), a kind of malignant tumor originating from the oral, pharynx, throat, and sinus mucosal epithelium [1], is the sixth most common cancer in the world [2], accounting for 4–7% of all malignancies [3, 4], with 890,000 deaths and even over 450,000 new cases in 2018 worldwide [5]. Despite advances in treatment, the 5-year overall survival rate of patients with HNSCC remains around 50% due to frequent metastasis and recurrence. Circular RNAs (circRNAs) have been characterized as key regulators of gene expression in numerous malignancies. However, the role of circRNA in HNSCC metastasis remains largely unknown. Here, we demonstrated that the circRFWD3 was significantly upregulated in HNSCC tissues and cell lines by circRNA microarray analysis and qPCR. Notably, high expression of circRFWD3 is related to highly aggressive HNSCC cell lines and lymph node metastasis in HNSCC patients. After that, Sanger sequencing, RNase R, and actinomycin D assay were performed to verify the ring structure of circRFWD3. Then functional experiments found it could promote the metastasis of HNSCC cells both in vitro and in vivo. Mechanistically, a dual-luciferase reporter assay, FISH, RIP, RNA pull-down, RNA-seq, and western blot experiments were employed and found that circRFWD3 served as a miRNA sponge for miR-27a/27b, leading to the upregulation of PPARγ, and then promoted HNSCC metastasis via NF-κB/MMP13 pathway. Finally, ISH and IHC were carried out to determine the expression levels and clinical significances of circRFWD3 and PPARγ in clinical cohorts of HNSCC. According to the analysis results from two independent HNSCC cohorts, upregulated expression of circRFWD3 and PPARγ were positively associated with worse survival in patients with HNSCC. Overall, our results uncover that circRFWD3 acts a critical role in promoting the aggressiveness of HNSCC cells and is a prognostic marker for the disease, indicating that circRFWD3 may act as a potential therapeutic target in HNSCC.

Cell Death Discovery (2022) 8:285; https://doi.org/10.1038/s41420-022-01066-6

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Head and neck squamous cell carcinoma is the sixth most common cancer in the world, the 5-year survival rate of patients with HNSCC is still about 50% due to frequent metastasis and recurrence. Circular RNAs (circular RNAs) have recently been developed as non-endogenous non-coding RNAs that originate from back-splicing of precursor mRNAs and are characterized by single-stranded and closed covalent loops without 5′-3′ polarity. Compared with their linear RNA counterparts, circRNAs are more resistant to exonucleases and more stable [10]. CircRNAs show important biological functions mainly by acting as microRNA sponges, regulating protein function or gene transcription, splicing, and translation [11]. Numerous studies have indicated that the circRNA/miRNA/mRNA signaling axis exerts multiple functions in many physiologic and pathologic conditions, especially in cancers [12]. Recently, several circRNAs have been intensively characterized in HNSCC [13–15], but a great deal of circRNAs have not been explored, and the exact mechanism of circRNAs in tumor metastasis remains to be further explored.

In this study, with the application of circRNA microarray, we identified a novel unique RFWD3-derived circRNA, hsa_circ_101877 (termed circRFWD3), which showed significant upregulation in HNSCC. We found that circRFWD3 was a proto-oncogenic circRNA that promoted the migration and invasion ability of HNSCC. In particular, circRFWD3 served as a miRNA sponge for miR-27a/b, resulting in upregulation of PPARγ and then promoting metastasis of HNSCC via the NF-κB/MMP13 pathway. CircRFWD3 and PPARγ were positively associated with metastasis and could predict a worse prognosis in patients with HNSCC. These findings indicated that circRFWD3 might function as a promising therapeutic marker for HNSCC.
RESULTS
CircRFWD3 is overexpressed in HNSCC tissues and cell lines

Nine pairs of HNSCC tissues were divided into groups of three and paired adjacent nontumor tissues were also divided into corresponding groups of three. Then a circRNA microarray was performed to identify significantly expressed circRNAs in HNSCC. The rows represent groups of HNSCC tissue samples (C, normal tissue; T, tumor tissue), and columns represent circRNAs' numerical ID obtained from circBase. The genomic loci of the RFWD3 gene and circRFWD3 were primarily transcribed from exons 7 and 8 of RFWD3, which were detected by qRT-PCR and validated by Sanger sequencing amplified with divergent primers.

CircRFWD3 is transcribed from the gene RFWD3 on chromosome 16, resulting from back-splicing of exons 7 and 8 with a spliced sequence length of 347 bp. Sanger sequencing further confirmed the back-splicing junction site of circRFWD3 (Fig. 1B). In addition, PCR analysis confirmed that divergent primers could amplify circRFWD3 from cDNA, but not gDNA (Fig. 1E). Furthermore, compared with normal tissues and cell lines, qRT-PCR assays illustrated prominently higher expression of circRFWD3 in HNSCC tissues and cell lines, which verified the upregulation of circRFWD3 expression in HNSCC (Fig. 1C, D). In particular, the higher expression of circRFWD3 was steadily shown in UM1 and HN31 cells (P < 0.0001), which were then chosen to perform the following experiments. RNase R digestion

Fig. 1 Screen and characterization of circRFWD3 in HNSCC. A Clustered heatmap showing the 32 differentially expressed circRNAs in three groups of human HNSCC tissues and adjacent normal tissues (red indicates upregulated circRNAs, and blue indicates downregulated circRNAs). The rows represent groups of HNSCC tissue samples (C, normal tissue; T, tumor tissue), and columns represent circRNAs’ numerical ID obtained from circBase. B The genomic loci of the RFWD3 gene and circRFWD3 were primarily transcribed from exons 7 and 8 of RFWD3, which were detected by qRT-PCR and validated by Sanger sequencing amplified with divergent primers. C, D The expression level of circRFWD3 in 43 normal tissues and 17 HNSCC tissues (data from TCGA) and in normal HOK cells and three HNSCC cell lines. E Agarose gel electrophoresis with RT-PCR products using divergent and convergent primers in both gDNA and cDNA. Red arrows showed that divergent primers could amplify circRFWD3 from cDNA. Red arrows showed that divergent primers could amplify circRFWD3 from cDNA. F, G qRT-PCR was used to determine the abundance of circRFWD3 and linear RFWD3 mRNA in UM1 and HN31 cells treated with RNase R. Results demonstrated circRFWD3 was tolerant to RNase R. H CircRFWD3 resistance to actinomycin D (ActD) was detected by the qRT-PCR assay. I RNA FISH showed that circRFWD3 located in the cytoplasm in UM1 and HN31 cells, scale bars, 10 μm. Data were shown as the mean value ± SD of three independent experiments. The asterisks indicate significant differences (Student’s t-tests, *P < 0.05, **P < 0.01, ***P < 0.001).
CircRFWD3 promotes the migration and invasion ability of HNSCC cells both in vitro and vivo

We noticed that the expression of circRFWD3 in high-metastatic UM1 cells was higher than that in low-metastatic UM2 cells, suggesting that circRFWD3 may be involved in tumor metastasis and promote tumor progression. As expected, circRFWD3 was also more abundant in metastasis-derived HN31 cells than in matched primary tumor-derived HN30 cells (Fig. 2A). In addition, we observed that the expression level of circRFWD3 was observably higher in HNSCC tissues extracted from patients with lymph node metastasis than in nonmetastatic HNSCC tissues (Fig. 2B). Then, two siRNAs were designed to silence circRFWD3 without influencing RFWD3 mRNA levels in HNSCC cells (Appendix Fig. 1A, B). si-circRFWD3-2 was chosen for the following intravitral investigations on account of its high inhibitory efficiency. The results of transwell assays showed that the migration and invasion abilities of UM1 and HN31 cells were remarkably restricted by the downregulation of circRFWD3 (Fig. 2C–F).

Next, to verify the circRFWD3-dependent on HNSCC cell metastasis in vivo, we adopted a lung metastasis mouse model. Anti-mitochondria, which is an excellent marker for human cells in xenograft model research, was used to detect metastasis in mouse lung tissues. The lungs of mice transplanted with sh-circRFWD3 rarely harbored metastatic tumor nodules, while the lungs of mice transplanted with sh-NC cells were widely occupied by visible metastatic tumors (Fig. 2G, H). These data distinctly showed that the decreased expression of circRFWD3 could visibly weaken the metastasis of HNSCC.

CircRFWD3 augments PPARγ/NF-κB/MMP13 signaling axis via sponging miR-27a/b in HNSCC

Subsequently, we treated HNSCC cells with the PPARγ inhibitor, GW9662 [21], and the western blot results showed that the expression of PPARγ was decreased (Fig. 6A). Transcripome analysis was then performed to identify the downstream targets mediated by PPARγ. A total of 20 mRNAs were upregulated and 66 mRNAs were downregulated in PPARγ-repressed UM1 cells, while 57 mRNAs were upregulated and 80 mRNAs were downregulated in PPARγ-repressed HN31 cells (Fig. 6B, C and Appendix Tables S5, 6). Considering that PPARγ should be positively correlated with HNSCC metastasis based on our existing data, we, therefore, focused on the downregulated genes. Among these differentially downregulated mRNAs, MMP13, STRA6, and PTPRH were downregulated in both HN31 and UM1 cells (Fig. 6D). Among them, MMP13 was most remarkably downregulated, belongs to the matrix metalloproteinase family, and contributes to tumor metastasis [22]. Furthermore, KEGG pathway enrichment analyses showed their significant enrichment in the IL-7 signaling pathway, which is involved in tumor migration and invasion, such as the NF-κB pathway and MAPK pathway in HNSCC cell lines (Appendix Fig. 3C, D). Western blot analysis indicated that inhibition of PPARγ decreased the expression levels of p65, p-p65, Rel B, and MMP13 (Fig. 6E and Appendix Fig. 3F), as well as MMP2 and MMP7 in UM1 and HN31 cells (Appendix Fig. 3E). The above results indicated that PPARγ could regulate the NF-κB/MMP13 pathway in HNSCC.
Additionally, we found that the protein levels of PPARγ were remarkably decreased in sh-circRFWD3-transfected HNSCC cells, and their expression could be restored by miR-27a or miR-27b inhibitors. Similarly, the protein levels of p65, p-p65, Rel B, and MMP13 were also significantly decreased in sh-circRFWD3-transfected UM1 and HN31 cells, and miR-27a and miR-27b inhibitors abolished their downregulation induced by sh-circRFWD3 (Fig. 6F). These findings demonstrated that circRFWD3 regulated the PPARγ/NF-κB/MMP13 signaling pathway via miR-27a/b in HNSCC.

CircRFWD3 and PPARγ were positively associated with metastasis and could predict a worse prognosis in patients with HNSCC

Using an HNSCC tissue microarray (TMA) consisting of 214 HNSCC tissues, we assessed the correlation of circRFWD3 or PPARγ
expression with clinicopathological characteristics by ISH or IHC (Fig. 7A, B). Further analysis showed that the expression levels of both circRFWD3 and PPARγ were significantly correlated with lymph node metastasis ($P = 0.013$ and $P = 0.004$, respectively, Tables 1, 2) of HNSCC. PPARγ expression was also positively correlated with the age ($P = 0.038$) and clinical TNM stage ($P = 0.000$) of HNSCC (Table 2). Furthermore, Kaplan–Meier analysis demonstrated that HNSSC patients with low expression levels of circRFWD3 or protein expression levels of PPARγ had a longer OS than those with high expression ($P < 0.0001$, Fig. 7C, D). Consistently, both the expression of circRFWD3 and the mRNA expression of PPARγ were negatively correlated with the overall survival of HNSSC patients from the TCGA database ($P = 0.0307$ and $P = 0.0053$, respectively, Fig. 7E, F). Therefore, circRFWD3 and PPARγ could serve as tumor promoters, and their high expression might predict a poor prognosis for HNSSC patients. These findings suggested that circRFWD3 and PPARγ could be potential targets for predicting the prognosis of patients with HNSSC.

**DISCUSSION**

HNSSC is the sixth most common malignant tumor in the world. The incidence is increasing year by year, and the prognosis is poor. Metastasis is one of the main reasons for the poor prognosis of HNSSC. However, due to the lack of effective therapeutic targets, HNSSC metastasis-targeted therapy has not yet made a major breakthrough. Therefore, it is urgent to elucidate the regulatory mechanism of HNSSC metastasis and discover valuable therapeutic
Fig. 4 Both miR-27a and miR-27b could reverse the tumor metastasizing-effects of circRFWD3 in HNSCC cells. A, C Transwell assays indicated that the migration ability of UM1 and HN31 cell were observably restricted by transfection of miR-27a and miR-27b mimics. B, D Transwell assays indicated that the invasive capabilities of UM1 and HN31 cell were observably restricted by transfection of miR-27a and miR-27b mimics. E–H Transwell assays revealed that miR-27a and miR-27b inhibitors could rescue the inhibitory effects of circRFWD3 knockdown of migration and invasion in UM1 and HN31 cells. Scale bar, 100 μm. Data were shown as the mean value ± SD of three independent experiments. The asterisks indicate significant differences (Student’s t-tests, *P < 0.05, **P < 0.01, ***P < 0.001).
targets. Here, we discovered a novel mechanism in HNSCC metastasis: circRFWD3 could sponge miR-27a/b to promote metastasis by augmenting PPAR\(\gamma\)/NF-\(\kappa\)B/MMP13 signaling (Fig. 7G).

CircRNAs, as a new type of noncoding RNAs, mainly have several characteristics: species conservation, unique circular and stable structures, cell and tissue specificity, and stable expression in saliva, blood, and extracellular vesicles. Increasing amounts of circRNAs have been detected and their biogenesis, characteristics, and functions have been explored in a variety of cancers [23, 24]. To date, despite rapid advances in studies based on circRNAs, little is known about the explicit roles of circRNAs in HNSCC, especially in the development and

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**Fig. 5** PPAR\(\gamma\) is a direct target of both miR-27a and miR-27b in HNSCC. A The network of target genes of miR-27a and miR-27b, the red circle represents PPAR\(\gamma\). B Schematic illustration showed binding site sequences between PPAR\(\gamma\) and miR-27a/27b. C RIP assay showed PPAR\(\gamma\) mRNA was effectively pulled down by AGO2 with a much greater enrichment in UM1 cells. D RNA pull-down assay exhibited for the direct bonding of PPAR\(\gamma\) and miR-27a/27b in UM1 cells. 

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**Table 1** Results of circRNA expression in HNSCC samples.

| CircRNA   | Expression Level |
|-----------|-----------------|
| circRFWD3 | High            |
| miR-27a   | Low             |
| miR-27b   | High            |

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**Fig. 6** Western Blot assay showed miR-27a and miR-27b mimic could significantly reduce expression of PPAR\(\gamma\) in UM1 and HN31 cells at the level of proteins.

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**Fig. 7** RNA FISH showed colocalization of PPAR\(\gamma\) and miR-27a/27b in UM1 and HN31 cells (Scale bar, 10 \(\mu\)m).
metastasis of HNSCC [25, 26]. In this study, we reported comprehensive circRNA expression profiles of HNSCC. According to our analysis and summary, a large number of circRNAs are abnormally expressed, among them, circRFWD3 was selected and characterized and exhibited an important function in regulating HNSCC metastasis.

CircRFWD3 is derived from the RFWD3 gene, which has a susceptible site for malignant neoplasms [27, 28]. In our study, we demonstrated that circRFWD3 originating from RFWD3 was abnormally overexpressed in HNSCC, and weakening circRFWD3 could inhibit the athletic ability of HNSCC cells. The most well-known function of circRNA is miRNA sponge in cancers [29]. Here, we identified that circRFWD3 contained conserved sites for two members of the miR27 family, miR-27a and miR-27b. Previous studies have reported the dual effect of cancer-promoting and cancer-inhibiting roles played by miR-27a and miR-27b in different cancers [30, 31]. Compared with previous findings, we reported for the first time that circRFWD3 exerts its functions in promoting tumor metastasis by harboring miR-27a/b to enhance the expression of PPARγ, which is the downstream target of miR-27a/b.

PPARγ is a member of the peroxisome proliferator-activated receptors, and its famous function is to regulate fat cell differentiation, body immunity, and insulin resistance [32]. The role of PPARγ in tumor development is controversial. On the one hand, on account of its proapoptotic and antiproliferative functions, PPARγ has been extensively considered a cancer suppressor, such as in colorectal cancer and breast cancer [33]. On the other hand, increasing evidence indicates that PPARγ acts as a cancer promoter. For example, PPARγ was reported to induce the production of reactive oxygen species to promote tumor growth in glioblastoma [34]. Different roles of PPARγ in different tumors, which may be due to organ-specific, considering that HNSCC also have organ specificity, we wanted to clarify the role of this molecule in HNSCC. In this study, we proved that PPARγ is negatively correlated with HNSCC prognosis and could promote metastasis of HNSCC, and the inhibition of endogenous PPARγ by reducing circRFWD3 or increasing miR-27a/b expression could restrain the migration and invasion ability of tumors.

The NF-κB signaling pathway is activated in multiple types of cancers and could play a role as a tumorigenesis promoter [35].

Fig. 6 CircRFWD3 regulated PPARγ/NF-κB/MMP13 signal pathway via miR-27a/27b in HNSCC. A Western blot showed that PPARγ expression was decreased after GW9662 treatment in HN31 cells. B, C Clustered heatmap of a significant differentially expressed gene in UM1 and HN31 cells treated by GW9662. Each sample contained a mixture of three repeats. D Venn diagram showed MMP13, STRA6, and PTPRH were downregulated after GW9662 treatment in both HN31 and UM1 cells. E Western blot analysis showed inhibition of PPARγ could decrease the expression level of p65, p-P65, Rel B, and MMP13. F Rescue analysis indicated that miR-27a and miR-27b inhibitors could alleviate the reduction of PPARγ, p65, p-P65, Rel B, and MMP13 by inhibiting the expression of circRFWD3. Data were shown as representative of one experiment with three independent biological replicates.
Fig. 7  The expression of circRFWD3 and PPARγ were negatively associated with prognosis in patients with HNSCC. A, B ISH staining of circRFWD3 (pink stain in the cytoplasm) and IHC staining of PPARγ in an HNSCC clinical cohort, scale bar, 10 μm. C, D Kaplan–Meier analysis showed that circRFWD3 and PPARγ were negatively correlated with the overall survival rate of HNSCC patients according to our clinical cohort. E, F Kaplan–Meier analysis showed that circRFWD3 and the mRNA expression of PPARγ were negatively correlated with the overall survival rate of HNSCC patients in the TCGA database. G Molecular mechanism of circRFWD3 involved in HNSCC metastasis. CircRFWD3, resulting from back-splicing of exons 7 and 8, could sponge-like bind to miR-27a/b to relieve the inhibitory effect of miR-27a/b on PPARγ, promoting the transcription and translation of PPARγ. Then, upregulated PPARγ could activate the NF-κB signaling pathway to promote the expression of MMP13 and accelerate tumor metastasis.
Rel B and p65 are two core members of the NF-κB family. It was reported that NF-κB suppression is beneficial for inhibiting tumor growth and metastasis in HNSCC [36]. The protein family of matrix metalloproteinases can degrade extracellular matrix components, whose well-known role is modulating tumor metastases, such as MMP2, MMP9, MMP7, MMP13, and MMP14 [37]. Previous reports showed that MMP13 could regulate tumor aggressiveness in oral cancer via the HBP1-MMP13 axis and can act as a diagnostic and prognostic molecular marker in HNSCC [38]. Because NF-κB is one of the transcription factors of MMP13, it is supposed that PPARγ could regulate MMP13 through NF-κB. As hypothetically, we demonstrated that PPARγ could regulate the NF-κB/MMP13 pathway. Furthermore, suppression of circRFWD3 suppressed the NF-κB pathway and the expression of MMP13 in HNSCC cells, while miR-27a and miR-27b inhibitors reversed this suppression. The data suggested that circRFWD3 enhanced the migration and invasion ability of HNSCC by weakening the expression of miR-27a/b, upregulating PPARγ, and then activating the NF-κB/MMP13 signaling axis.

**CONCLUSION**

In summary, our findings suggested the presence of the circRFWD3/miR-27a/b/PPARγ/NF-κB/MMP13 axis in regulating HNSCC metastasis, highlighting the potential of circRFWD3 as a novel therapeutic target in HNSCC metastasis. Furthermore, the clinical expression patterns of circRFWD3 and PPARγ suggested that they may be promising prognostic biomarkers and therapeutic targets for HNSCC.

**METHODS**

*Patients and follow-up*

A total of 9 pairs of tumors and adjacent nontumor tissues and 30 cases of metastatic and nonmetastatic samples were collected from pathologically
diagnosed patients with HNSCC (Appendix Table S1). Noteworthily, a cohort of 214 HNSCC pathological samples and related clinical information was obtained (Tables 1, 2). Data on the other cohort comprising 499 patients with HNSCC, were obtained from The Cancer Genome Atlas (TCGA), EGA, and GEO database. The study was approved by the ethics committees of the West China Hospital of Stomatology, Sichuan University, and was conducted in agreement with the Helsinki Declaration. Written informed consent was provided by all participants at baseline and during follow-up.

CircRNA microarray analysis

Nine tumor tissues were divided into groups of three and paired adjacent nontumor tissues were also divided into corresponding groups of three. Then, a total of six groups of tissues were deployed for circRNA microarray (Arraystar Inc.) and analyzed using Arraystar Human circRNA Array V2. Raw sequencing data of circRNAs have been uploaded to GEO (GSE200946). Overall, the assay indicated a total of 3157 circRNAs, among them, 59 circRNAs were stably upregulated and 76 circRNAs were stably downregulated in the three groups in comparison with nontumor tissues. Then, we screened the candidates according to the following strategies: 200 bp ≤ the length of the base ≤ 1500; fold change ≥2 and P < 0.05. After validation in circBase, 32 circRNAs were obtained (Appendix Table S2).

Cell cultures

Detailed information on HOK, UM1, UM2, HSC-3, CAL27, HN12, HN31, HN30, H413, and HEK293T cells is shown in Appendix Table S3. HOK cells were cultured in a defined keratinocyte SFM medium (10744019, Thermo Fisher Scientific). UM1, UM2, HSC-3, CAL27, HN12, HN31, H413, and HEK293T cells were appropriately cultured in DMEM (SH30243.01, HyClone) supplemented with 10% FBS and 1% penicillin-streptavidin solution. All cells were regularly tested for mycoplasma with a MycAwayTM Plus-Color One-Step Mycoplasma Detection Kit (40612ES25, Yeasen). Cells were kept in a humidified incubator at 37°C with 5% CO₂.

| Characteristics          | Number of Cases | PPARγ (Low) | PPARγ (High) | p valuea |
|--------------------------|-----------------|-------------|--------------|----------|
| Age at surgery           |                 |             |              |          |
| ≤60 years                | 94              | 29          | 65           | 0.038    |
| 60 years                 | 120             | 40          | 80           | 0.900    |
| Gender                   |                 |             |              |          |
| Male                     | 154             | 51          | 103          | 0.866    |
| Female                   | 60              | 18          | 42           | 0.585    |
| Smoking                  |                 |             |              |          |
| No                       | 102             | 30          | 72           |          |
| Yes                      | 112             | 39          | 73           |          |
| Alcohol consumption      |                 |             |              |          |
| No                       | 105             | 34          | 71           |          |
| Yes                      | 109             | 35          | 74           |          |
| Differentiation          |                 |             |              |          |
| High                     | 136             | 46          | 90           |          |
| Moderate                 | 56              | 20          | 36           |          |
| Poor                     | 22              | 3           | 19           |          |
| High vs. moderate + poor |                 |             |              | 0.304    |
| Tumor stage              |                 |             |              |          |
| T1                       | 48              | 14          | 34           |          |
| T2                       | 103             | 36          | 67           |          |
| T3                       | 20              | 4           | 16           |          |
| T4                       | 43              | 15          | 28           |          |
| T1-2 vs. T3-4            |                 |             |              | 0.000    |
| Clinical TNM stage       |                 |             |              |          |
| I                        | 32              | 8           | 24           |          |
| II                       | 70              | 25          | 45           |          |
| III                      | 67              | 23          | 44           |          |
| IV                       | 45              | 13          | 32           |          |
| I–III vs IV              |                 |             |              | 0.955    |
| Lymph node metastasis    |                 |             |              |          |
| No                       | 137             | 47          | 90           |          |
| Unilateral               | 70              | 21          | 49           |          |
| Bilateral                | 7               | 1           | 6            |          |
| No vs. unilateral + bilateral |           |             |              | 0.004    |

*a P values were derived using the Spearman rank correlation coefficient test; all statistical tests are two-sided.

The expression levels of PPARγ were significantly correlated with lymph node metastasis (P = 0.004), the age (P = 0.038), and clinical TNM stage (P = 0.000) of HNSCC according to our clinical cohort (214 cases of HNSCC).
Oligonucleotide transfection
Six-well plates were used to culture UM1 and HN31 cells, and transduction was carried out when cells were 70–80% confluent. Serum-free medium was used before transduction reagents were added to the corresponding plates. Cells were transfected using Lipofectamine 2000 (11668019, Invitrogen Lipofectamine 2000) following the product’s manual, and cells were seeded after being cultured for 48–72 h. All siRNAs targeting circRFWD3 and miRNA mimics or inhibitors were synthesized by Ribobio (Guangzhou, China).

Plasmids
An expression construct encoding shRNA of circRFWD3 was cloned into pGPU6/Neo (GenScript, USA) to produce pGPU6/sh-circRFWD3 recombinant lentivirus (sh-circRFWD3). Empty pGPU6 lentivirus (sh-NC) was used as a control. The pmir-27a/b-Luc plasmid was constructed by amplification of the upstream 2000 bp to downstream 100 bp sequence of mir-27a/b and subcloning into the pGL3-Basic plasmid. The wild-type or point-mutated 3’ UTR of PPAR-γ 1 and the invaded or migrated cells were counted in five randomly selected fields of view at 400x. The experiment was replicated three times.

Stable cell line generation
GV341-sh-circRFWD3 recombinant lentiviruses (sh-circRFWD3 and sh-NC) were purchased from NeuronBiotech (Shanghai Genechem Co., Ltd.). UM1 and HN31 cells were infected with sh-circRFWD3 and sh-NC. Selective culture medium containing 15 μg/ml puromycin was used to select the cells with stable expression of low circRFWD3 or vector controls. The expression of circRFWD3 was detected by RT-qPCR.

RNA isolation and qRT-PCR
Total RNA was isolated using RNA Pure kits (TR-205-50, ZYMO RESEARCH) according to the manufacturer’s guidelines. The concentration and purity of each RNA extract were checked at a 260/280 ratio on a NanoDrop Microvolume UV-Vis Spectrophotometer instrument (Thermo Fisher Scientific, One®). Complementary DNA (cDNA) synthesis was achieved by using the PrimeScript™ RT Reagent Kit (RR037A, TaKaRa) according to the manufacturer’s guidelines. Quantitative PCR was performed with SYBR™ Select Master Mix (4472908, Applied Biosystems) following the product’s instructions. Primer sequences were listed in Appendix Table S4. Three technical replicates were set for every single reaction to ensure reliability and validity. The ΔΔCT value of the target gene expression was measured and assessed against the value of the reference genes GAPDH and U6.

RNase R digestion treatment and Sanger sequencing
RNase R digestion treatment was performed by incubating 4 μg of total RNA mixture at 37°C for 10 min with or without 3 U/μg of RNase R, as suggested by the product’s manual (RNR 07250, Epicenter). Following the removal of linear forms, qRT-PCR qualification was performed to confirm the circular structure of circRFWD3. Sanger sequencing covering the back-splicing junction sequence was performed using the qRT-PCR product of circRFWD3 by TSINGKE company. A total of 2% agarose gel was used for DNA electrophoresis. The sequences of divergent primers and convergent primers are shown in Appendix Table S4.

Actinomycin D assay
UM1 and HN31 cells were exposed to a complete medium (DMEM with 1 μg/ml actinomycin D (A1410, Sigma-Aldrich) to block transcription for 0, 2, 4, 6, 8, 10, and 12 h. The cells were harvested and the expression of circRFWD3 and RFWD3 mRNA was analyzed using qRT-PCR.

Cell invasion assay
UM1 and HN31 cells at a concentration of 1 × 10^5 cells in 200 μl of serum-free medium were inoculated in the upper chamber and coated with growth factor-reduced Matrigel® (356234, Corning), and a medium containing 15% FBS was added to the lower chamber as a chemotractant. After incubation for 24 h (UM1) or 30 h (HN31), cells on the upper surface of the membrane and cells were removed by wiping with a Q-tip, and the invaded or migrated cells were fixed with formaldehyde and stained using 0.5% crystal violet. The numbers of migrated and invaded cells were counted in five randomly selected fields under a microscope. The experiment was replicated three times.

Cell migration assay
The assay was similar to the in vitro invasion assay, except that no growth factor-reduced Matrigel® (356,234, Corning) was added to the chamber. After incubation for 18 h (UM1) or 24 h (HN31), cells on the upper surface of cells were removed by wiping with a Q-tip, and the invaded or migrated cells were fixed with formaldehyde and stained using 0.5% crystal violet. Cells in five randomized fields of view at 400x were counted and represented as the average number of cells per field of view. The experiment was also replicated three times.

RNA FISH
RNA fluorescence in situ hybridization (FISH) was performed using the Fluorescent in situ Hybridization Kit (Inc1CM001, Ribobio) according to the manufacturer’s instructions. Cy3-labeled probes targeting circRFWD3, SFAM-labeled mir-27a/27b, and Cy3-labeled probes targeting PPARγ were synthesized by Ribobio (Guangzhou, China). Fluorescence was recorded with a confocal laser scanning microscope (FV3000, OLYMPUS).

RNA pull-down assay
RNA pull-down assays were performed according to the Pierce™ Magnetic RNA-Protein Pull-Down Kit (20164, Thermo Scientific). Biotinylated circRFWD3, miR-27a, and miR-27b probes were synthesized by Ribobio (Guangzhou, China). Pull-down RNA released from the beads after cleansing was evaluated by qRT-PCR.

RNA immunoprecipitation
RNA immunoprecipitation (RIP) experiments were performed with a RIP-Assay Kit (RN001, BML) according to the manufacturer’s guidelines. Briefly, UM1 cells were collected and resuspended in 200 μl RIPA lysis buffer. A total of 100 μl of each cell lysate was incubated with Protein G Agarose Beads (37478 S, Cell Signaling Technology) conjugated with 15 μl argonaute 2 (Anti-EIF2C2 (AGO2) (Human) mAb, RN003 M, BML) or control rabbit IgG protein with gentle agitation at 4°C overnight. Immunoprecipitated RNA was then purified and measured by qRT-PCR for enrichment.

Western blot analysis
For Western blot analysis, cells were washed three times with 1x PBS and then used for extraction of total proteins. Protein extracts were prepared by mammalian lysis buffer. Protein concentrations were measured by the Pierce™ BCA Protein Assay Kit (23250, Thermo Scientific). Protein extracts were separated by 10% SDS page and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, IPVH00010). Then, the PVDF membranes were blotted individually with appropriate primary antibodies against GAPDH (2148 S, Cell Signaling Technology, dilution rate 1:3000), PPARγ (2435 S, Cell Signaling Technology, dilution rate 1:1000), p65 (8242 S, Cell Signaling Technology, dilution rate 1:1000), p-p65 (3033 S, Cell Signaling Technology, dilution rate 1:1000), Rel B (ab33917, Abcam, dilution rate 1:1000), MMP13 (ab51072, Abcam, dilution rate 1:1000), MMP9 (40094 S, Cell Signaling Technology, dilution rate 1:1000), MMP7 (3801 S, Cell Signaling Technology, dilution rate 1:1000), and appropriate secondary antibodies (mouse, ZB-2305; rabbit, ZB2301, ZSGB-BIO, dilution rate 1:3000). Protein bands were visualized using a chemiluminescence system (Amersham Imager 600).

Luciferase reporter assay
Wild-type and predicted binding site-specific mutated mir-27a, mir-27b, and PPARγ 3’ UTR sequences were synthesized and integrated into psiCHECK 2.0 vectors and cotransfected into HEK293T cells with mir-27a or mir-27b mimics using Lipofectamine 2000 (11668019, Invitrogen Lipofectamine 2000). HEK293T cells were lysed, and luciferase intensity was measured 24 h after transfection. The ratio of Renilla luciferase to firefly luciferase absorbance was calculated to quantitatively analyze the interaction between miR-27a and miR-27b and PPARγ using the luciferase reporter assay protocol recommended by Promega (E1910, Promega, USA).

In situ hybridization (ISH)
ISH experiments were performed with an ISH-Assay Kit (329900, BaseScope Reagent Kit v2-RED) according to the manufacturer’s guidelines. The circRFWD3 detection probe sequence (BaseScope Probe-BA-Hs-RFWD3-E8E7-circRNA-Junc) was 5’-TCCCTACTGAGAAGACATGCCTAAAGGAAACGACG-3’.
dislocation. The time point for sacri-
two criteria: loss of >15% of pre-injection body weight and/or when the
contains 12 mice (12). Specifi-
cally, sh-circRFWD3 or sh-NC-infected
UM1 and HN31 cells were lysed with TRIzol
reagent (15596026, Thermo Fisher), and total RNA was extracted according
to the standard method. Genes with [fold change [2 and P < 0.05] were
considered differentially expressed. Next, RNA sequencing was performed,
and target genes were analyzed.

Animal experiments
Forty-eight female BALB/c nude mice (SFP level) aged 5–6 weeks were
purchased from Charles River Company (Beijing, China), which were
raised and manipulated in the Animal Central Laboratory of West China
Second Hospital in compliance with the institutional guidelines for animal
use and care. Mice were randomly divided into four groups: UM1 sh-
circRFWD3, UM1 sh-NC, HN31 sh-circRFWD3, and HN31 sh-NC. Each group
contains 12 mice (n = 12). Specifically, sh-circRFWD3 or sh-NC-infected
UM1 and HN31 cells (Appendix Fig. 1C–F) were intravenously injected
to the tail vein of mice to construct the lung metastasis mouse model.
Caudal vein injection was performed with 100 μL stroke-physiological
saline solution suspension of 10⁷ cells using a 1 mL insulin syringe. After
injection, two mice died in 24 h because of cardiac failure and were
excluded. Animal weight was monitored weekly following injection.
Euthanasia was initiated by CO₂ suffocation and finished by cerebral
dislocation. The time point for sacrifice was based on a combination of
two criteria: loss of >35% of pre-injection body weight and/or when the
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Kaplan–Meier curves and log-rank test for significance. P values of <0.05
were considered statistically significant. The data were the mean ± SD of
three experiments. (*P < 0.05, **P < 0.01, ***P < 0.001).

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ACKNOWLEDGEMENTS

This work was supported by grants from the National Natural Science Foundation of China (81872211, 82072999, U19A2005, 81991500, and 81991502), the 111 Project of MOE China (grant B14038), CAMS Innovation Fund for Medical Sciences (2020-I2M-CBT-A-023 and 2019-I2M-5-004), and 13-5 project of excellent development of the discipline of West China Hospital of Sichuan University (No. ZYYC21001), and the Sichuan Science and Technology Program (2020YJ0102).

AUTHOR CONTRIBUTIONS

Zihao Wei, Ying Wang, and Jing Li contributed to data acquisition, analysis and interpretation, and drafted and critically revised the manuscript. Jiakuan Peng, Honglin Li, Junjie Gu, Ning Ji, and Taiwen Li contributed to the design, data acquisition, analysis, drafted, and critically revised the manuscript. Jing Li, Xin Zeng, Xikun Zhou, and Qianming Chen contributed to the conceptualization, funding acquisition, drafted, and critically revised the manuscript. All authors gave their final approval and agreed to be accountable for all aspects of the work.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41420-022-01066-6.

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