Received: 22 April 2022 | Revised: 15 July 2022 | Accepted: 20 July 2022

DOI: 10.1111/cas.15505

ORIGINAL ARTICLE

Cancer Science WILEY

Transfer RNA-derived fragment 5′tRF-Gly promotes the development of hepatocellular carcinoma by direct targeting of carcinoembryonic antigen-related cell adhesion molecule 1

Dekai Liu[1,2,3,4,5,6] | Chengdong Wu[1,2,3,4,5,6] | Jingjie Wang[1,2,3,4,5,6] | Lufei Zhang[1,2,3,4,5,6] | Zhongquan Sun[1,2,3,4,5,6] | Shihong Chen[7] | Yuan Ding[1,2,3,4,5,6] | Weilin Wang[1,2,3,4,5,6]

[1]Department of Hepatobiliary and Pancreatic Surgery, The Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China

[2]Key Laboratory of Precision Diagnosis and Treatment for Hepatobiliary and Pancreatic Tumor of Zhejiang Province, Hangzhou, China

[3]Research Center of Diagnosis and Treatment Technology for Hepatocellular Carcinoma of Zhejiang Province, Hangzhou, China

[4]Clinical Medicine Innovation Center of Precision Diagnosis and Treatment for Hepatobiliary and Pancreatic Disease of Zhejiang University, Hangzhou, China

[5]Clinical Research Center of Hepatobiliary and Pancreatic Diseases of Zhejiang Province, Hangzhou, China

[6]Cancer Center, Zhejiang University, Hangzhou, China

[7]Department of Clinical Medicine, Wenzhou Medical College, Wenzhou, China

Correspondence
Weilin Wang and Yuan Ding, Hepatobiliary and Pancreatic Surgery, The Second Affiliated Hospital, School of Medicine, Zhejiang University, No. 88 Jiefang Road, Hangzhou, Zhejiang 310009, China.
Emails: wam@zju.edu.cn (W.W.); dingyuan@zju.edu.cn (Y.D.)

Funding information
National Natural Science Foundation of China, Grant/Award Number: 81773096, 82072650 and 81902405; Health Commission of Zhejiang Province, Grant/Award Number: JBZX-202004; 2019 Liver Cancer Diagnosis and Treatment Communication Fund, Grant/Award Number: CXPJJH11900009-12; Key Research and Development Program of Zhejiang Province, Grant/Award Number: 2021C03121 and 2018C03085

Abstract

Transfer RNA-derived fragments are a group of small noncoding single-stranded RNA that play essential roles in multiple diseases. However, their biological functions in carcinogenesis are not well understood. In this study, 5′tRF-Gly was found to have significantly high expression in hepatocellular carcinoma (HCC), and the upregulation of 5′tRF-Gly was positively correlated with tumor size and tumor metastasis. Overexpression of 5′tRF-Gly induced increased growth rate and metastasis in HCC cells in vitro and in nude mice, while knockdown showed the opposite effect. Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) was confirmed to be a direct target of 5′tRF-Gly in HCC. In addition, the cytological effect of CEACAM1 knockdown proved to be similar to the overexpression of 5′tRF-Gly. Moreover, attenuation of CEACAM1 expression rescued the 5′tRF-Gly-mediated promoting effects on HCC cells. These data show that 5′tRF-Gly is a new tumor-promoting factor and could be a potential diagnostic biomarker or new therapeutic target for HCC.

KEYWORDS
5′tRF-Gly, biomarker, CEACAM1, hepatocellular carcinoma, tRNA-derived small RNA

Abbreviations: CEACAM1, carcinoembryonic antigen-related cell adhesion molecule 1; EMT, epithelial to mesenchymal transition; GO, Gene Ontology; HCC, hepatocellular carcinoma; LCSC, liver cancer stem cell; LNA, locked nucleic acid; NAFLD, nonalcoholic fatty liver disease; NATs, nontumor adjacent tissue specimen; NC, negative control; PFS, progression-free survival; qPCR, quantitative PCR; tRF, transfer RNA-derived fragment; tiRNA, transfer RNA-derived stress-induced RNA; tsRNA, transfer RNA-derived small RNA.

Dekai Liu and Chengdong Wu contributed equally to this article.

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1 | INTRODUCTION

Hepatocellular carcinoma is still identified as one of the most widely diagnosed cancers worldwide. With an age-adjusted worldwide incidence of 10.1 cases per 100,000 person-years, HCC is ranked as the sixth most common neoplasm and the third leading cause of cancer death.[1] The incidence and mortality of HCC in China are as high as 17.64 per 100,000 and 15.33 per 100,000, respectively,[2] meaning that HCC still poses a serious challenge for Chinese people. Hence, in-depth study of the molecular mechanism of HCC occurrence and development is helpful for the early diagnosis of HCC patients and could provide a new therapeutic target for HCC treatment.

In recent years, with the advances in RNA sequencing technology, multiple noncoding RNAs have been discovered, and tsRNAs have gradually attracted attention. Previously, tsRNAs were considered to be useless degradation products of tRNA. However, current research described these molecules as a new class of noncoding RNA rooted in tRNAs, and many studies have shown that they play a certain regulatory role in multiple cellular activities.[3] According to the characteristics of the splicing site, they are classified into two main groups: tRF and tRNA halves (also called tiRNA).[4–6] Misregulated tsRNAs exert different effects in various cancer types, such as ovarian,[7,8] breast,[9–12] bladder,[13] and liver cancer,[14,15] due to their involvement in diverse cellular events, including epigenetic regulation, gene transcription, and mRNA processing.[16]

Generated from the 5′-arm of glycine tRNA, 5′tsRNA-Gly, is implicated in the pathological processes of multiple diseases. A recent study has illustrated that 5′tiRNA-Gly could promote cell proliferation and migration through binding to RBM17 in papillary thyroid cancer.[17] In addition, it has been reported that expression of 5′tRFs-Gly is upregulated in ethanol-fed mice and promotes chronic ethanol feeding-induced hepatosteatosis.[18] These findings aroused our curiosity about its function in HCC.

In the present study, the small RNA high-throughput sequencing results showed that 5′tRF-Gly was upregulated in HCC tissues and was related to poor prognosis. We determined the oncogenic role of 5′tRF-Gly and its underlying molecular mechanism in HCC. Furthermore, we first verified CEACAM1 as the direct target of 5′tRF-Gly and revealed how CEACAM1 inhibits HCC growth and metastasis. Our findings not only elucidate the role of 5′tRF-Gly and CEACAM1 in HCC but also provide new targets for the prognostic evaluation and treatment of HCC.

2 | MATERIALS AND METHODS

2.1 | Human samples

We obtained four pairs of HCC tissue samples and matched nontumor adjacent tissues from patients who underwent surgical resection in the Second Affiliated Hospital of Zhejiang University. Tissues were stored at −80°C for further high-throughput sequencing. Seventy-seven paired HCC specimens with clinicopathologic diagnosis were also acquired from the Second Affiliated Hospital of Zhejiang University Specimen Bank. Ethics approval for the study was obtained from the Second Affiliated Hospital of Zhejiang University Ethics Committee.

2.2 | High-throughput sequencing

TRIzol (Invitrogen) was used to isolate total RNA, and RNA quantity was assessed using NanoDrop 2000 (Thermo Fisher Scientific). After RNA pretreatment with the rtStar tRF & tiRNA Pretreatment Kit (Arraystar), the small RNA library was prepared using the NEBNext Multiplex Small RNA Library Prep Set for Illumina (NEB), according to the instructions of the manufacturer. The library was sequenced using the Illumina NextSeq 500 according to the procedures of the NextSeq 500/550 V2 kit (Illumina). EdgeR was used to analyze tRFs changes across groups.

2.3 | Cells and cell culture

The HCC cell lines HepG2, Hep3B2.1-7, and SK-HEP-1 were maintained in minimum essential medium, supplemented with 10% FBS (Hyclone). The Huh-7 and 293T/17 cells were maintained in DMEM with 10% FBS (Hyclone). In contrast, Li-7 cells were maintained in RPMI-1640 (Gibco) supplemented with 10% FBS (Hyclone). All cell lines were purchased from WuHan Procell Life Science & Technology.

2.4 | Plasmid construction

For the luciferase assay, the sequence of a 200-bp segment containing the predicted 5′tRF-Gly binding site on the 3′-UTR of CEACAM1 was amplified by PCR and cloned into the *XhoI* and *NotI* sites of the psiCHECK-2 dual-luciferase reporter vector (Promega). Site-direct mutagenesis of the predicted 5′tRF-Gly binding site on the 3′-UTR of CEACAM1 was synthesized by GenePharma. All constructs were verified by sequencing.

2.5 | Oligonucleotide and plasmid transfection

Oligonucleotide and plasmid transfection was undertaken with Lipofectamine 3000 (Invitrogen). Negative control mimics and 5′tRF-Gly mimics were synthesized by Takara and negative control LNA, LNA-5′tRF-Gly, and siRNA for CEACAM1 were synthesized by GenePharma. The final concentration of 67 nM mimics was optimized for 35-mm culture dishes. For plasmid transfection, the final concentration was 1 μg/ml. The oligonucleotide sequences are listed in Table S1.

2.6 | RNA isolation and RT-qPCR

The total RNA of fresh tissues and cells was isolated using TRIzol (Invitrogen). RNA quality and quantity were measured by a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Selected tRF expression was evaluated using TaqMan-based qPCR. The specific stem-loop RT primers and probes were purchased from GenePharma; the sequences are listed in Table S2. Reverse transcription-qPCR was carried out using a Roche LightCycler 480 II system. The relative tRF expression was normalized to human U6 levels and analyzed by the comparative threshold cycle ($2^{-\Delta\Delta Ct}$) method. For mRNA transcripts, total RNA was directly reverse-transcribed into cDNA with random primers. The expression level was determined using TB Green (Takara) and normalized by GAPDH. The mRNA primers are listed in Table S2. The fold changes were calculated with the $2^{-\Delta\Delta Ct}$ method.

2.7 | Luciferase activity analysis

Luciferase reporter assay was undertaken on 293T/17 cells. After overnight incubation, the cells at 70% confluence were cotransfected with appropriate oligonucleotides and plasmid transfection. Whole-cell lysates were prepared 24 h after transfection, and the luciferase activity was measured using Dual-Luciferase Reporter Gene Assay Kit (Yeasen). The *Renilla* luciferase activity was normalized to that of firefly luciferase.

2.8 | Cell proliferation assay

The CCK-8 assay was used to detect the cell proliferation rate. Cells were seeded in 96-well plates at a density of 4000/well. After incubation overnight, the cells were transfected with appropriate oligonucleotides. Subsequently, 10 μl CCK-8 reagent was added to each well and incubated for 2 h at 37°C after transfection for the indicated times. Cell proliferation was assessed on days 1–4 after seeding using optical density values at 450 nm.

2.9 | Transwell migration and invasion assays

The migration and invasion assays were completed in Transwell chambers (8 μm pore size; Millipore). For the migration assay, cells were washed twice with serum-free growth medium, and 1×10^5 cells were seeded in chambers and cultured for 24 h in 24-well plates (BD Biosciences) containing 600 μl 10% serum medium as a migratory stimulant. Cells that migrated through the chamber were fixed in 4% paraformaldehyde and stained with crystal violet (Beyotime). Images were captured, and cell numbers were counted with a microscope (Nikon). For the invasion assay, the upper chambers were coated with 80 μl Matrigel plug (BD Biosciences) at a final concentration of 250 ng/ml at 37°C for 2 h. The subsequent steps followed the methods of the migration assay. Finally, the cell count was repeated in three representative fields for each independent experiment.

2.10 | Animal study

Cells were infected with lentiviral-5′tRF-Gly or lentiviral-WT according to the manufacturer's instructions (GeneChem). The Hep3B2.1-7 cells stably overexpressing 5′tRF-Gly were selected by incubating with puromycin (3 μg/ml) for 2 days and were validated by RT-qPCR. For in vivo pulmonary metastasis assay, male BALB/c nu/nu mice (four mice per group) were injected through the lateral tail vein with 1×10^6 cells stably overexpressing 5′tRF-Gly or WT RNA. After 6 weeks, all the mice were killed and the lungs were collected. After the process of paraffin embedding, the lung samples were sectioned and stained with H&E. For in vivo tumorigenesis assay, male BALB/c nu/nu mice (five mice per group) were injected subcutaneously with 5×10^6 cells stably overexpressing 5′tRF-Gly or WT RNA. After 4 weeks of feeding, the mice were killed. The tumors were then collected, measured, and weighed.

2.11 | Western blot analysis

Cells were lysed with RIPA lysis buffer. All samples were resolved by SDS-PAGE, transferred to nitrocellulose membranes (Whatman), and probed with the appropriate Abs. The Abs used in this study were rabbit anti-CEACAM1 (ab108397; Abcam), rabbit anti-N-cadherin (22018-1-AP; Proteintech), mouse anti-MMP2 (66366-1-IG; Proteintech), mouse anti-E-cadherin (60335-1-IG; Proteintech), mouse anti-Cyclin D1 (60186-1-IG; Proteintech).

2.12 | RNA immunoprecipitation assay

The Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore) was used to undertake RIP assays. Cells were lysed by the NP-40 lysis buffer containing PMSF, DTT, protease inhibitor cocktail, and RNase inhibitor. The cells were then incubated with RIP buffer containing magnetic beads bound with human anti-Argonaute2 Ab (Millipore) or normal mouse IgG (Millipore) as a negative control for 4 h at 4°C. Precipitate was digested with proteinase K buffer, and then coimmunoprecipitated RNA was isolated for RT-qPCR analysis.

2.13 | Statistical analysis

All statistical analyses and graphs were carried out with GraphPad Prism 6.0. The difference between two groups was assessed using Student's *t*-test. Data are presented as the mean and SEM. The difference between variables was evaluated by the χ^2-test. *p* values less than 0.05 were considered to be statistically significant.

3 | RESULTS

3.1 | 5′tRF-Gly overexpression indicates poor prognosis in HCC

Following high-throughput sequencing of four pairs of HCC clinical tissues, we screened four upregulated (log2 fold change > 3, $p < 0.001$) and five downregulated (log2 fold change < −3, $p < 0.001$) tRFs in tumor tissues between the two groups (Figure 1A). We undertook RT-qPCR on these four highly expressed tRFs in eight pairs of HCC tissues. We identified a 22-nt sequence (5′-GCATGGGTGGTTCAGTGGTAGA-3′) that was most significantly differentially expressed in tumors compared with NATs (Figure 1B), and chose it for in-depth research. At least five perfect matches for this fragment were obtained in MINTbase version 2.0,[19] corresponding to the 5′-end of the respective annotated human tRNAs. The cleavage site was located on the D-loop of these sequences (MINTbase-ID: tRF-22-P4R8YP9LL). The candidate mature tRNA carried the amino acid glycine by the anticodon GCC (Figure 1C), so we named it 5′tRF-Gly. The results of tiRNA profiling suggested that alterations in 5′tRF-Gly could be involved in HCC progression. To test this conjecture, our sample number was expanded to 77 pairs of HCC tissues evaluated with RT-PCR. The results indicated that 5′tRF-Gly had significantly higher expression in HCC compared with NATs (Figure 1D,E). Furthermore, higher expression of 5′tRF-Gly was also significantly correlated with larger tumor size (diameter < 7 cm vs. ≥ 7 cm: $p < 0.001$) and tumor metastasis ($p < 0.001$) in HCC patients (Figure 1F,G, Table 1, χ^2-test). A borderline significant correlation was observed for higher 5′tRF-Gly expression and poorly differentiated tumor (Table 1, $p = 0.076$, χ^2-test). Likewise, the OncotRF database[20] showed that the overall survival of the high 5′tRF-Gly (OncotRF-ID: 5′-M-tRNA-Gly-GCC-1-5_L22) expression group was significantly shorter than that of the low expression group in HCC patients (Figure 1H, $p = 0.00547$). Therefore, we reasoned that 5′tRF-Gly could act as a tumor promoter to enhance HCC progression.

3.2 | 5′tRF-Gly promotes HCC cell proliferation, migration, and invasion in vitro

The expression levels of 5′tRF-Gly in different HCC cell lines were determined using RT-qPCR, and we found that 5′tRF-Gly was highly expressed in Hep3B2.1-7 cells, but Huh7 cells featured weak expression (Figure 2A). Thus, we decided to use Huh7 cells and Hep3B2.1-7 cells to investigate the biological role of 5′tRF-Gly in vitro. We transfected the 5′tRF-Gly mimics and NC into HCC cells. Locked nucleic acid technology[21] was used to block the intracellular 5′tRF-Gly. In addition, we used RT-qPCR to confirm the overexpression and inhibition of 5′tRF-Gly in HCC cells (Figure 2B). The CCK-8 assay showed that overexpression of 5′tRF-Gly resulted in an increased growth rate of HCC cells, while inhibition led to opposite findings (Figure 2C). Transwell assays indicated that the migration and invasion ability of HCC cells increased after 5′tRF-Gly mimics transfection and decreased after LNA-5′tRF-Gly transfection (Figure 2D,E). Cell proliferation did not significantly change after transfection with 5′tRF-Gly mimics or LNA-5′tRF-Gly under serum-free culture conditions (Figure S1A,B). Therefore, the impact of proliferation on invasion and migration could be ruled out. Together, these results indicate that 5′tRF-Gly acts as a tumor-promoting factor to enhance the proliferation, migration, and invasion of HCC cells in vitro.

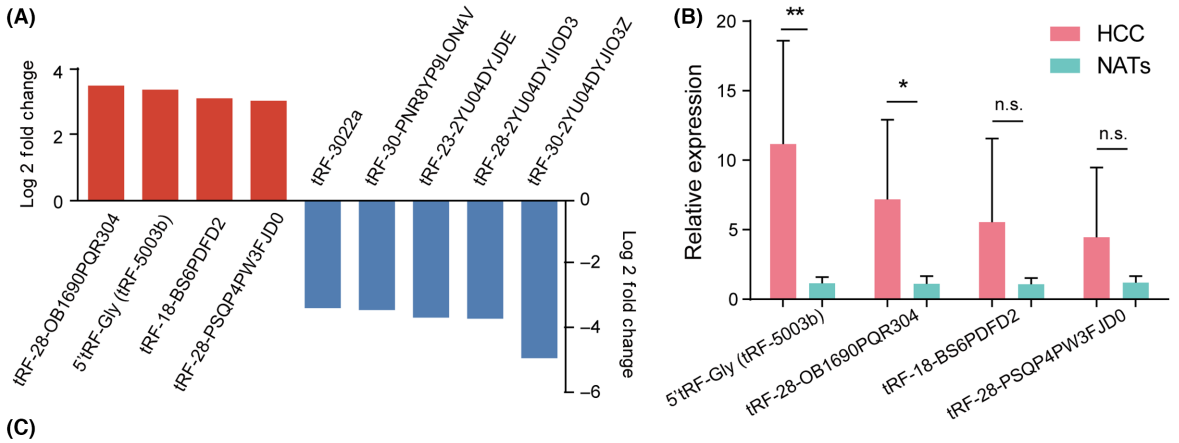
3.3 | 5′tRF-Gly enhances growth and migration of HCC cells in vivo

To verify the in vivo effect of 5′tRF-Gly, we constructed a lentiviral expression vector that stably overexpresses 5′tRF-Gly in Hep3B2.1-7 cells. Reverse transcription-qPCR showed that 5′tRF-Gly expression was significantly overexpressed following lentiviral infection (Figure 3A). We established lung metastasis mouse models, and 5′tRF-Gly overexpressed cells (Lenti-5′tRF-Gly) or control WT cells (Lenti-WT) were transplanted into nude mice by lateral tail vein injection. After 6 weeks, the mice were killed, and the lungs were dissected and fixed for H&E staining (Figure 3B). The results proved that 5′tRF-Gly overexpression significantly promoted lung metastasis (Figure 3C). Moreover, the two groups of cells were injected subcutaneously into each flank of nude mice. The xenografted tumors were collected after 4 weeks (Figure 3D). As observed in Figure 3E,F, tumors from the Lenti-5′tRF-Gly group grew faster and resulted in a larger size and a heavier weight than those from the Lenti-WT group. Collectively, these results indicate that 5′tRF-Gly enhances growth, migration, and invasion of HCC cells in vivo.

3.4 | 5′tRF-Gly directly regulates CEACAM1 expression in HCC cells

Previous studies have revealed an miRNA-like function for tRF by binding to the mRNA 3′-UTR of target genes and inducing gene silencing.[22,23] Therefore, the miRNA target gene prediction software

FIGURE 1 Transfer RNA-derived fragment (tRF) 5′tRF-Gly overexpression indicates poor prognosis in hepatocellular carcinoma (HCC). (A) Differential expression of tRF sequencing in four pairs of HCC and nontumor adjacent tissue specimens (NATs). (B) Relative expression of four highly expressed tRFs in eight pairs of HCC tissues. (C) This sequence is a 5′tRF of tRNA-Gly-GCC, and matched to five annotated human tRNAs. (D) Expression of 5′tRF-Gly in 77 pairs of samples from HCC and NATs. (E) Paired comparison of 5′tRF-Gly expression levels between HCC and NATs (tumor/normal). (F) Percentages of HCC samples with upregulated 5′tRF-Gly in tumor diameter (d) <7 cm and ≥7 cm tissues. (G) Percentages of HCC samples with upregulated 5′tRF-Gly in metastasis and nonmetastasis tissues. (H) Higher expression of 5′tRF-Gly is related to poorer prognosis in liver hepatocellular carcinoma (LIHC) on the OncotRF database. *$p < 0.05$. n.s., not significant



(C)

MINTbase v2.0: a database for the interactive exploration of mitochondrial and nuclear tRNA fragments (tRFs)

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Sequence	GCATGGGTGGTTCAGTGGTAGA (Print summary)
MINTbase Unique ID (sequence derived)	tRF-22-P4R8YP9LL
Available genomes	*H. sapiens* (hg19/GRCh37)
MINTbase Alternative IDs (GRCh37 assembly-derived)	trna35_GlyGCC_1_+_161413094_161413164@1.22.22 trna37_GlyGCC_1_+_161420467_161420537@1.22.22 trna39_GlyGCC_1_+_161427898_161427968@1.22.22 trna41_GlyGCC_1_+_161435258_161435328@1.22.22 trna2_GlyGCC_21_-_18827107_18827177@1.22.22
Genomic locations	5 (View in genomic loci vista)
Type(s)	5'-tRF (View in RNA molecule vista)

-1 nucleotide | D loop | Anticodon loop | Anticodon | intron | T loop | CCA tail

Candidate mature tRNA sources		
NGCATGGGTGGTTCAGTGGTAGAATTCTCGCCTGCCACGCGGGAGGCCCGGGTTCGATTCCCGGCCCATGCANNN	View tRNA alignment	tRNA-Gly-GCC-1-1 View summary
NGCATGGGTGGTTCAGTGGTAGAATTCTCGCCTGCCACGCGGGAGGCCCGGGTTCGATTCCCGGCCCATGCANNN	View tRNA alignment	tRNA-Gly-GCC-1-2 View summary
NGCATGGGTGGTTCAGTGGTAGAATTCTCGCCTGCCACGCGGGAGGCCCGGGTTCGATTCCCGGCCCATGCANNN	View tRNA alignment	tRNA-Gly-GCC-1-3 View summary
NGCATGGGTGGTTCAGTGGTAGAATTCTCGCCTGCCACGCGGGAGGCCCGGGTTCGATTCCCGGCCCATGCANNN	View tRNA alignment	tRNA-Gly-GCC-1-4 View summary
NGCATGGGTGGTTCAGTGGTAGAATTCTCGCCTGCCACGCGGGAGGCCCGGGTTCGATTCCCGGCCCATGCANNN	View tRNA alignment	tRNA-Gly-GCC-1-5 View summary

5'tRF-Gly

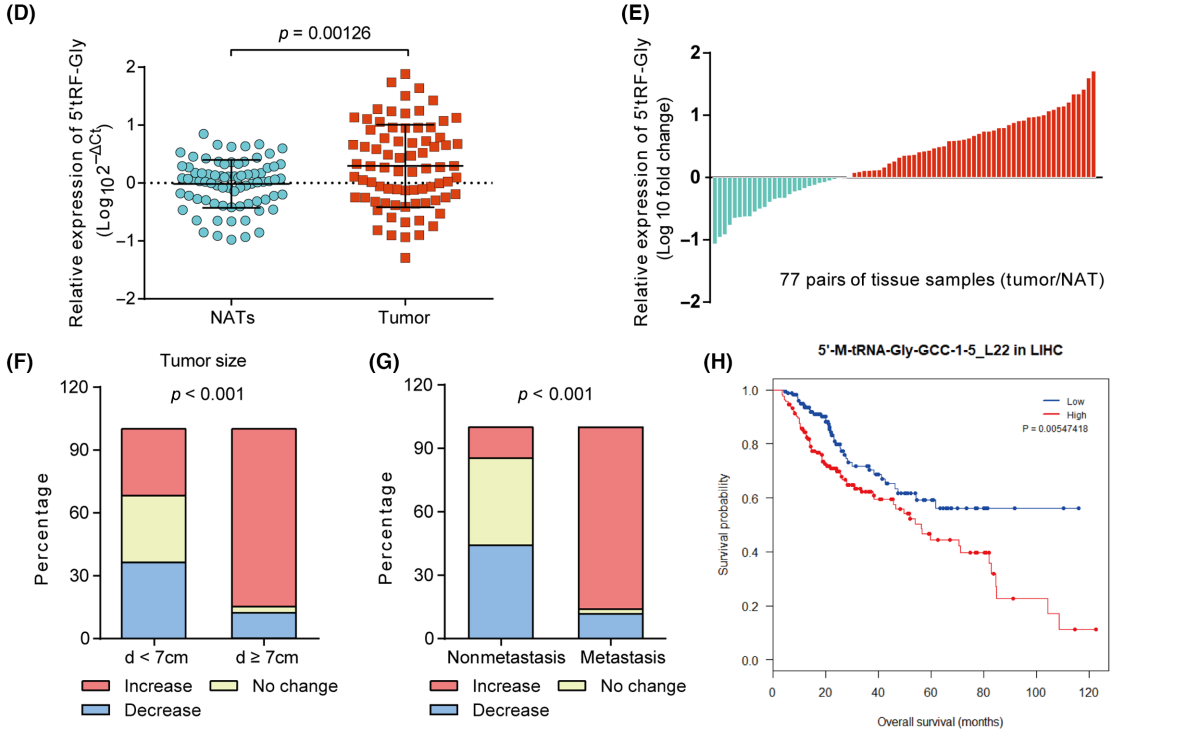

TABLE 1 Correlation between 5′tRF-Gly expression and clinicopathologic characteristics in patients with hepatocellular carcinoma

Characteristic	*N*	5′tRF-Gly decreased (FC ≤ 0.75)	5′tRF-Gly no change (0.75 < FC < 1.5)	5′tRF-Gly increased (FC ≥ 1.5)	χ^2-test	*p* value
Number	77	20	15	42		
Gender						
Male	68	18	13	37	0.096	0.953
Female	9	2	2	5		
Age (years)						
<50	19	2	3	14	4.188	0.123
≥50	58	18	12	28		
Number of tumors						
Single	69	16	15	38	3.757	0.153
Multiple	8	4	0	4		
Tumor size (cm)						
D < 7	44	16	14	14	22.011	<0.001
D ≥ 7 cm	33	4	1	28		
Metastasis						
No	34	15	14	5	40.144	<0.001
Yes	43	5	1	37		
Tumor differentiation						
Well/moderate	47	15	12	22	5.151	0.076
Poor	30	5	3	20		

Abbreviation: D, diameter.

miRanda and RNAhybrid were used to predict the target genes of 5′tRF-Gly in *Homo sapiens*. Two hundred and sixty-eight genes were identified by selecting the intersection of the target prediction software (Figure 4A). Then GO enrichment analysis was carried out using DAVID. Genes associated with cell growth regulation, cell junction, and cell–cell adhesion were selected for further investigation. Venn diagram analysis revealed that two genes (*SNX9* and *CEACAM1*) appeared in all three groups and three overlapping genes (*BCL2L1*, *CHRM1*, and *JUP*) appeared among the three pairwise comparisons (Figure 4A). To confirm the predicted target genes, 5′tRF-Gly mimics were transfected into HCC cells, and the expression of the predicted target genes was analyzed after 48 h. We found that the mRNA level of CEACAM1 decreased after transfection with 5′tRF-Gly mimics (Figure 4B). The cell expression of CEACAM1 was further validated by western blot analysis after transfection with 5′tRF-Gly mimics or LNA-5′tRF-Gly (Figure 4C).

The predicted binding sites for 5′tRF-Gly in the 3′-UTR of CEACAM1 and the mutations in the binding sites are shown in Figure 4D. A dual-luciferase reporter assay system was used to validate whether 5′tRF-Gly directly recognizes the 3′-UTR region of CEACAM1 mRNA. Our results revealed that 5′tRF-Gly mimics could suppress the luciferase activity of the wild form of pisCHECK-2-CEACAM1-3′-UTR, whereas there was no effect on the luciferase activity of the three CEACAM1 mutant reporter plasmids (Figure 4E). We further undertook RIPA to explore the binding status between 5′tRF-Gly and CEACAM1. Results showed that 5′tRF-Gly and CEACAM1 were both enriched in Ago2-coating beads relative to the IgG group. After transfected with 5′tRF-Gly mimics, we confirmed CEACAM1 is upregulated compared with the NC group by RIPA. The opposite changes of CEACAM1were also observed in Hep3B2.1-7 cells transfected with LNA-5′tRF-Gly (Figure 4F). These results indicated that 5′tRF-Gly inhibits the translation of CEACAM1 by directly targeting the 3′-UTR.

3.5 | Silencing of CEACAM1 promoted migration and proliferation of HCC cells

Recent studies have shown that CEACAM1 is downregulated in HCC compared to healthy tissue specimens.[24] Its overexpression can inhibit the cell proliferation of hepatocarcinoma cells and induce hepatocyte apoptosis in fulminant hepatic failure.[25,26] The progression-free survival of HCC was found to be related to the expression level of CEACAM1. Low expression of CEACAM1 was significantly associated with poorer PFS of HCC based on the Kaplan–Meier plotter database (Figure 5A). All the above results suggested that CEACAM1 could serve as a tumor suppressor in HCC cell tumorigenesis.

Small interfering RNA (siCEACAM1) was used to evaluate the cytological function of CEACAM1 for HCC cells. The western blot results and qPCR results both showed that the expression of CEACAM1 was significantly decreased after siCEACAM1 treatment (Figure 5B). As illustrated in Figure 5C, the CCK-8 assay showed that CEACAM1 silencing significantly increased the proliferation of HCC

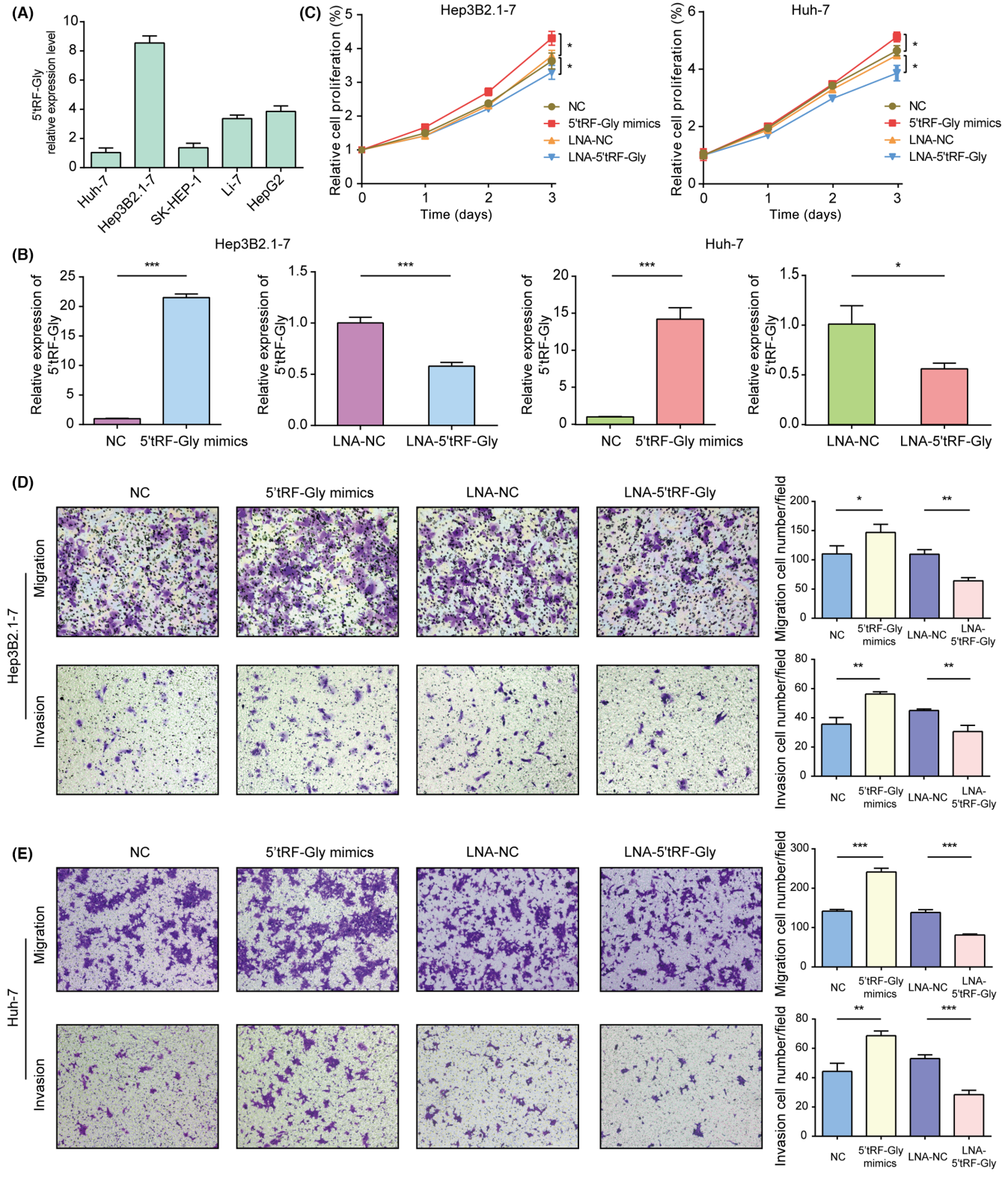

FIGURE 2 Transfer RNA-derived fragment 5′tRF-Gly promotes hepatocellular carcinoma (HCC) cell proliferation, migration, and invasion in vitro. (A) Quantitative real-time PCR (RT-qPCR) shows the expression level of 5′tRF-Gly in five kinds of HCC cell lines, normalized to Huh-7. (B) Confirmation of 5′tRF-Gly enhancement or reduction transfected with 5′tRF-Gly mimics or locked nucleic acid (LNA)-5′tRF-Gly by RT-qPCR in Hep3B2.1-7 and Huh-7 cells. (C) CCK-8 assays showing that overexpression of 5′tRF-Gly increased the viability of Hep3B2.1-7 and Huh-7 cells while inhibition of endogenous 5′tRF-Gly weakened it. (D, E) Representative images and quantification of Transwell migration and invasion assays after Hep3B2.1-7 (D) or Huh-7 cells (E) were transfected with negative control (NC), 5′tRF-Gly mimics, LNA-NC, or LNA-5′tRF-Gly. Magnification, ×100. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.0001$

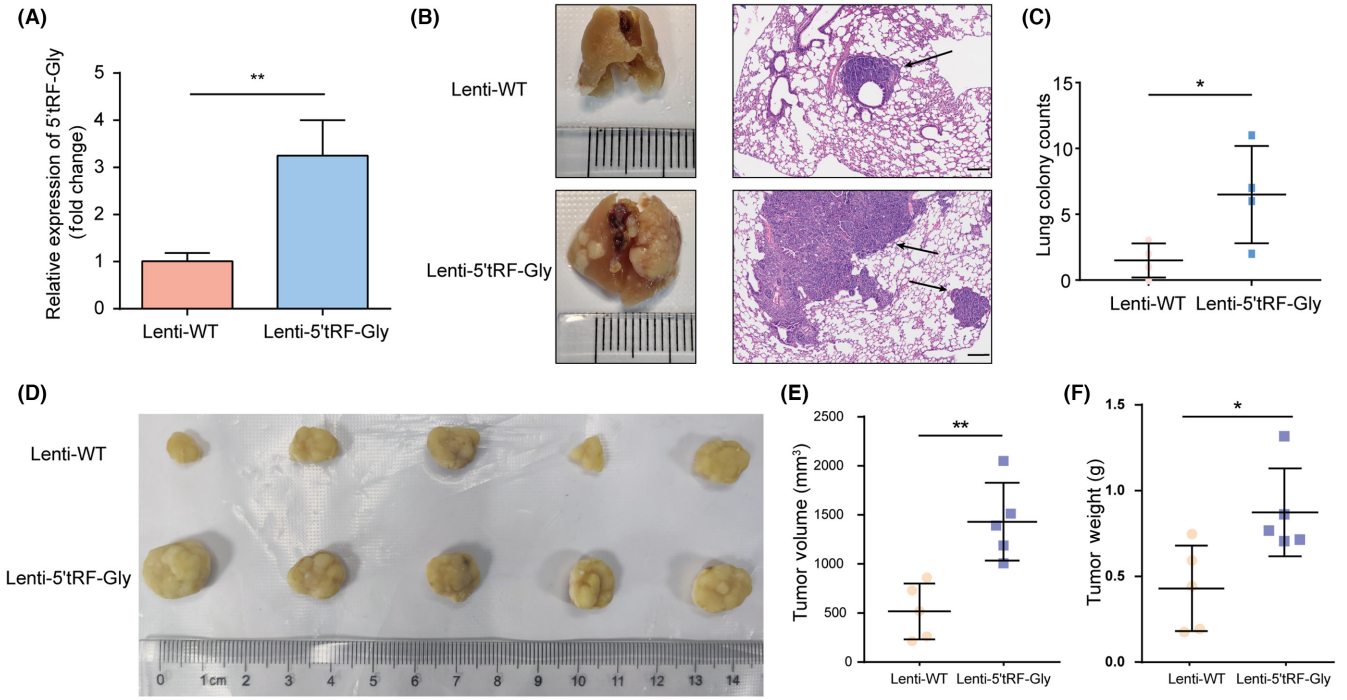

FIGURE 3 Transfer RNA-derived fragment 5′tRF-Gly enhances growth and migration of hepatocellular carcinoma cells in vivo. (A) Quantitative real-time PCR shows the expression of 5′tRF-Gly in Hep3B2.1-7 cells after lentiviral (Lenti) infection. (B) Representative images of H&E-stained sections of lung tissues isolated from the Hep3B2.1-7-WT and Hep3B2.1-7-5′tRF-Gly groups in pulmonary metastasis assay. Arrows indicate tumorous nodules. Scale bar, 200 μm. (C) Counts of lung metastasis in mice from the Hep3B2.1-7-WT and Hep3B2.1-7-5′tRF-Gly groups. (D) Images of tumors from nude mice injected subcutaneously with Hep3B2.1-7 cells infected with Lenti-WT or Lenti-5′tRF-Gly. (E, F) Tumor volume (E) and tumor mass (F) of xenografts from the Lenti-WT or Lenti-5′tRF-Gly groups. $^{*}p<0.05$; $^{**}p<0.01$

cells. Similar to the effect of 5′tRF-Gly, CEACAM1 knockdown markedly increased the migration and invasion of HCC cells in Transwell studies (Figure 5D). Similarly, we excluded the effect of cell proliferation on invasion and migration after transfection with siCEACAM1 or siNC under serum-free culture conditions (Figure S1C).

3.6 | Attenuation of CEACAM1 expression rescues 5′tRF-Gly-mediated effects on HCC cells

As established above, 5′tRF-Gly downregulates CEACAM1 expression by directly targeting its 3′-UTR. Subsequently, we investigated whether the role of 5′tRF-Gly on tumor progression was mediated by downregulation of CEACAM1. Therefore, we undertook a "rescue" experiment to investigate the effect of 5′tRF-Gly with or without CEACAM1 silencing by using Hep3B2.1–7 cells As shown in Figure 6A, cotransfection with CEACAM1 siRNA markedly abolished the elevated levels of CEACAM1 induced by the LNA-5′tRF-Gly in Hep3B2.1-7 cells. Moreover, results showed that cell proliferation, migration, and invasion were significantly downregulated following 5′tRF-Gly lockdown and was reversed by cotransfection with siCEACAM1 (Figure 6B–D). These data suggest that 5′tRF-Gly exerts its effects by inhibiting CEACAM1 in HCC cells.

Nevertheless, how CEACAM1 suppressed HCC growth, invasion, and migration remains unknown. As a reference, Leung et al. found increased cyclin D1 expression in CEACAM1 KO mice. The downregulation of CEACAM1 in intestinal tissue could contribute to malignancy by augmenting tumor multiplicity.[27] In addition, Xu et al.[28] discovered that CEACAM1 overexpression inhibited multiple myeloma cell metastasis through downregulation of MMP2. Liu et al.[29] described that CEACAM1 functions as an adhesion inhibitor and migration promoter by regulating cell-matrix adhesion. Thus, we further explored the probable underlying mechanism by focusing on the above-mentioned genes that might be regulated by CEACAM1.

We undertook western blot analysis of those genes for validation on cells cotransfected with LNA-5′tRF-Gly and si-CEACAM1. Results showed that the expression of MMP2 and cyclin D1 was obviously suppressed after 5′tRF-Gly locked, which was significantly reversed by cotransfection with siCEACAM1. The opposite effect is observed with E-cadherin (Figure 6E). In addition, those proteins were also detected after transfection with LNA-5′tRF-Gly or 5′tRF-Gly mimics in Hep3B2.1-7 cells These results further confirmed the effects of 5′tRF-Gly on regulating MMP2, cyclin D1, and E-cadherin (Figure 6F). Together, we can conclude that 5′tRF-Gly directly regulates the mRNA and protein expression of CEACAM1, further affecting the protein expression of downstream genes like MMP2, cyclin D1, and E-cadherin.

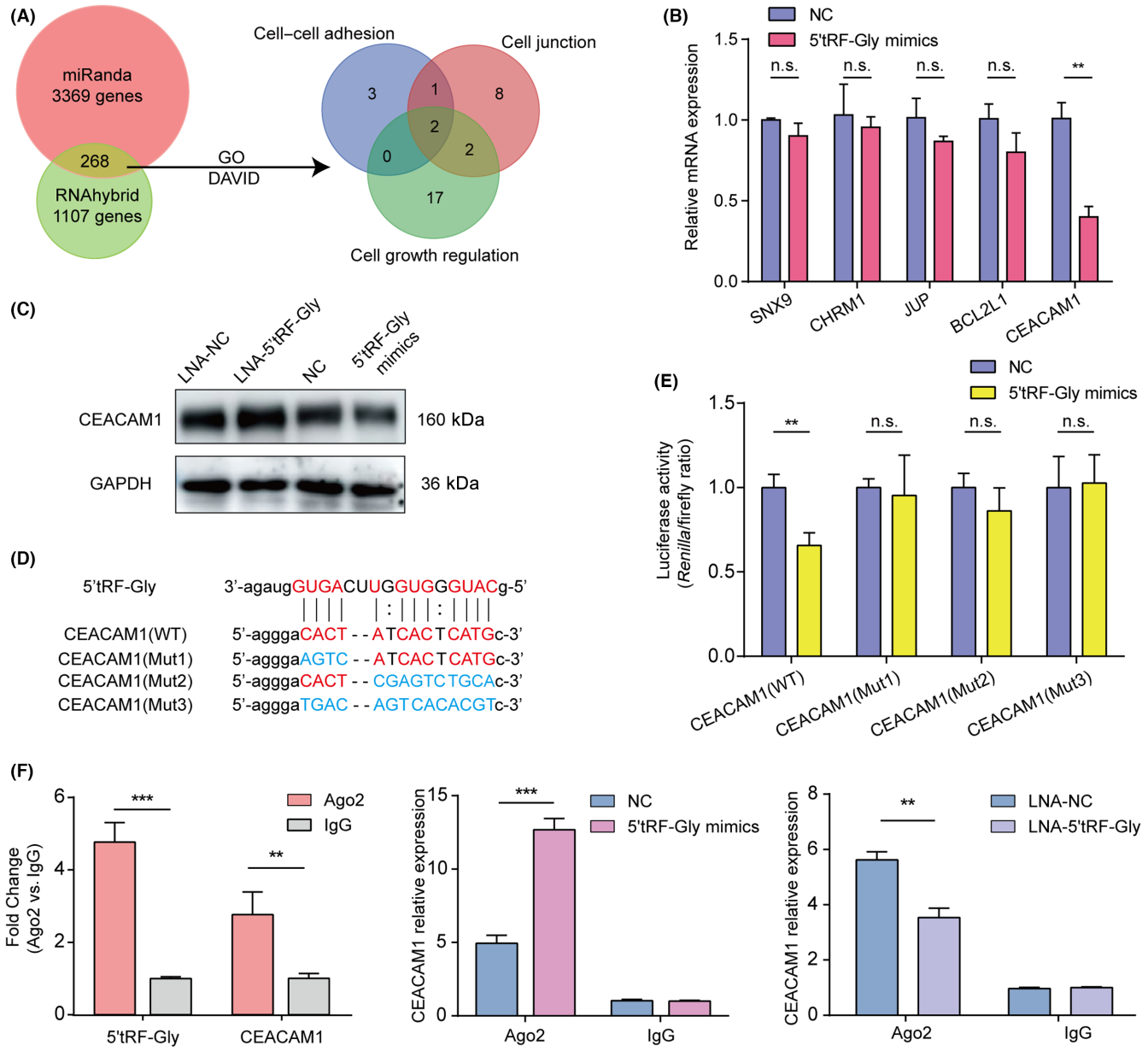

FIGURE 4 Transfer RNA-derived fragment 5′tRF-Gly directly regulates carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) expression in hepatocellular carcinoma cells. (A) Schematic illustration displaying the overlapped target genes of 5′tRF-Gly by miRanda and RNAhybrid. Five genes were selected by Gene Ontology (GO) enrichment analysis of cell growth, cell junction, and cell–cell adhesion. (B) mRNA expression of five target genes was tested by quantitative PCR in Hep3B2.1-7 cells after transfection with 5′tRF-Gly mimics. (C) Protein expression of CEACAM1 was tested in Hep3B2.1-7 cells after transfection with negative control (NC), 5′tRF-Gly mimics, locked nucleic acid (LNA)-NC, or LNA-5′tRF-Gly by western blotting. (D) Sequence of binding target of 5′tRF-Gly in WT or mutant CEACAM1 3′-UTR. (E) After transfection with NC or 5′tRF-Gly mimics in 293T/17 cells, the relative luciferase activity of three kinds of mutant or WT CEACAM1 3′-UTR was detected. (F) RIPA confirmed the binding status between 5′tRF-Gly and CEACAM1 in untreated and treated Hep3B2.1-7 cells. *$p<0.05$; **$p<0.01$; ***$p<0.0001$. n.s., not significant

4 | DISCUSSION

Recently, tsRNA has received significant attention. More and more studies have proven that abnormal expression of tsRNA is closely related to the development of diseases. Existing studies have implied that tsRNA is associated with cancer progression, but the effects of tsRNAs on different tumors are heterogeneous. LeuCAG3′tsRNA was reported to be highly expressed in mouse liver cancer cells and promote tumor growth by regulating ribosome biogenesis.[21] Some tsRNAs were shown to be tumor suppressors in breast cancer[9] and chronic lymphocytic leukemia.[30] However, studies on tsRNAs in HCC are relatively limited. Zhu et al.[31] reported four exosomal tsRNAs showing significantly high levels in liver cancer, but the possible mechanism has not been elaborated, and further research is needed.

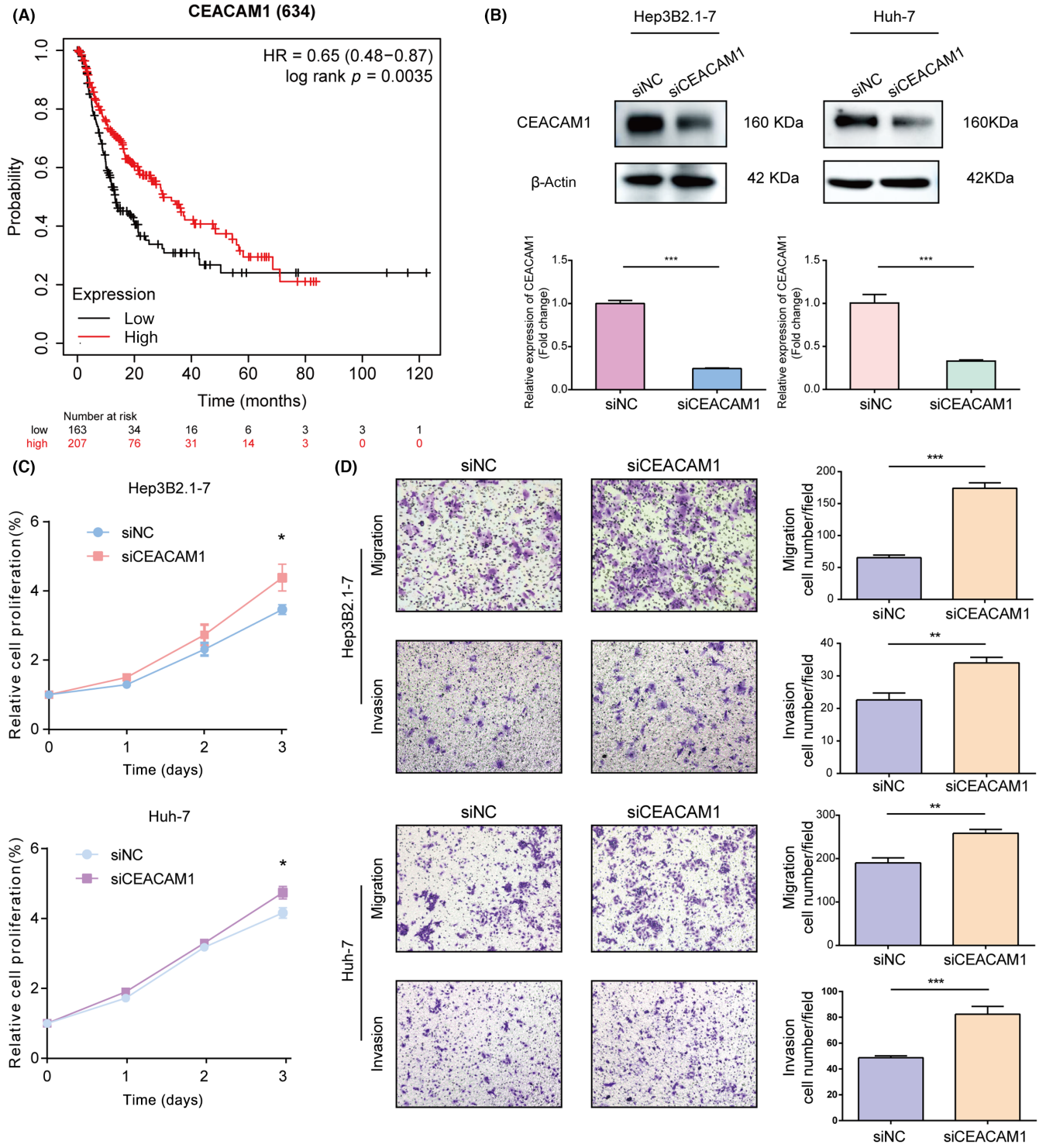

FIGURE 5 Silencing carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) promotes the proliferation, migration, and invasion of hepatocellular carcinoma (HCC) cells. (A) Higher expression of CEACAM1 is related to longer progression-free survival of HCC in the Kaplan–Meier Plotter database. (B) Expression level of CEACAM1 protein and mRNA in Hep3b2.1-7 and Huh-7 cells after transfection with siRNA of CEACAM1. (C) Proliferation rate of Hep3B2.1-7 and Huh-7 cells after CEACAM1 knockdown. (D) Representative images and quantification of Transwell migration and invasion assays after Hep3B2.1-7 or Huh-7 cells were transfected with negative control (siNC) or siCEACAM1. Magnification, ×100. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.0001$

Importantly, we identified a tRF, 5′tRF-Gly, that was elevated in HCC tissue and showed that high 5′tRF-Gly expression was related to poor prognosis in HCC patients. We uncovered the function and mechanism of 5′tRF-Gly in HCC. Meanwhile, CEACAM1 was also described as a tumor suppressor in HCC. Furthermore, MMP2, cyclin D1, and E-cadherin were confirmed as downstream genes of

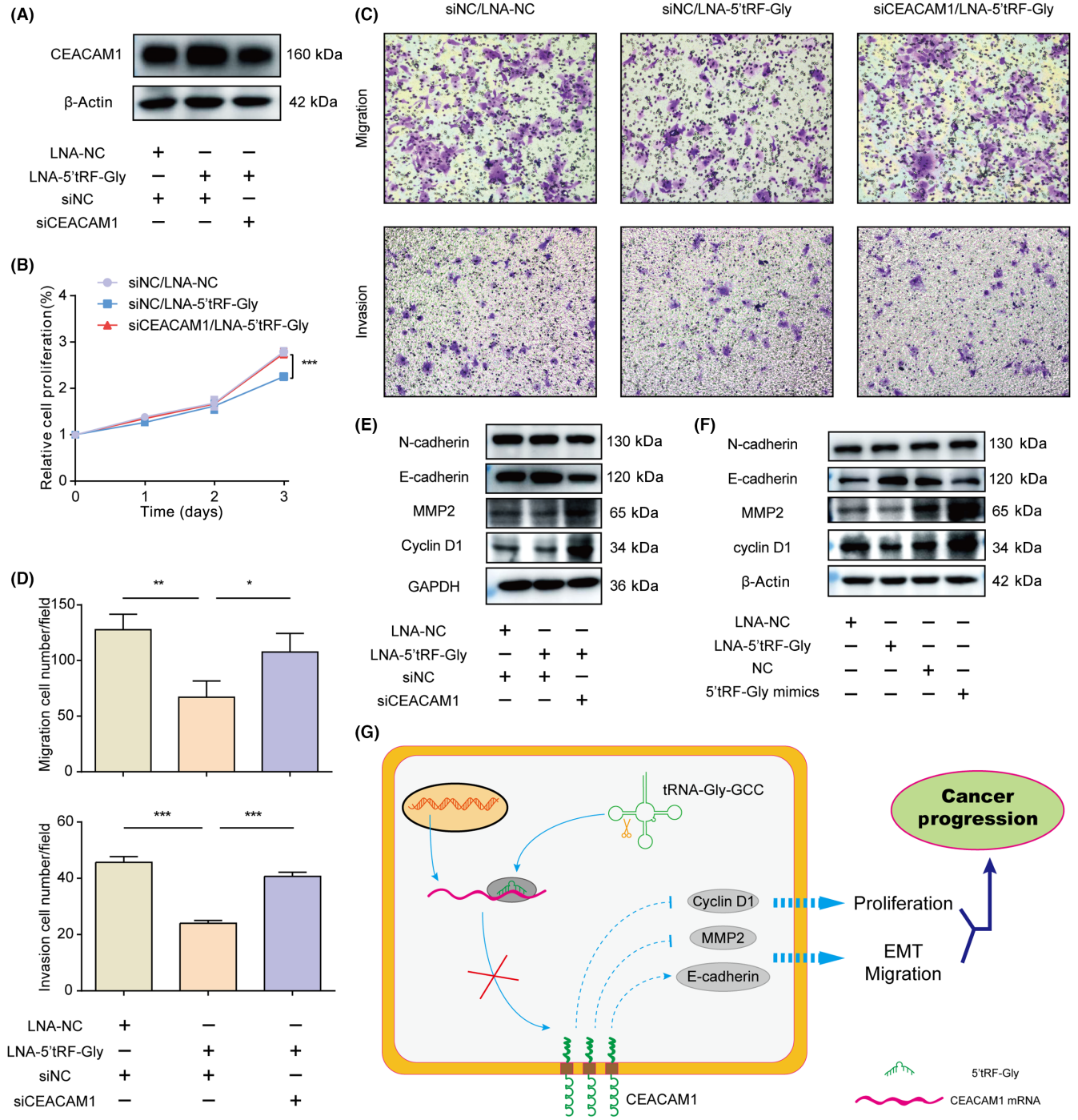

FIGURE 6 Role of transfer RNA-derived fragment 5′tRF-Gly on tumor progression is mediated by the downregulation of carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1). (A) Alteration of CEACAM1 after cotransfection with locked nucleic acid (LNA)-5′tRF-Gly or negative control (LNA-NC) with either CEACAM1 or NC siRNA. (B) Proliferation of Hep3B2.1-7 cells after cotransfection with LNA-5′tRF-Gly or LNA-NC with either CEACAM1 or NC siRNA. (C, D) Representative images (C) and quantification (D) of Transwell migration and invasion assays after Hep3B2.1-7 cells were cotransfected with LNA-5′tRF-Gly or LNA-NC with either CEACAM1 or NC siRNA. (E) Protein expression of the possible downstream genes of CEACAM1 were tested in Hep3B2.1-7 cells after cotransfection with LNA-5′tRF-Gly or LNA-NC with either CEACAM1 or NC siRNA by western blot analysis. (F) Alterations of possible downstream genes of CEACAM1 in Hep3B2.1-7 after transfection with NC, 5′tRF-Gly mimics, LNA-NC, or LNA-5′tRF-Gly. (G) Model diagram of 5′tRF-Gly/CEACAM1 axis functioning in HCC. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.0001$

CEACAM1. Together, these data reveal that 5′tRF-Gly could be a novel tumor promoter in HCC and that targeting tRF could be a promising treatment in HCC.

Coincidentally, we note that Zhou et al.'s study[15] found that another tRF derived from tRNA-Gly has a promoting effect on HCC cell migration by targeting NDFIP2. The sequence we researched

(MINTbase-ID: tRF-22-P4R8YP9LL) has a different source of mature tRNA than the Gly-tRF in their article (MINTbase-ID: tRF-23-PNR8YP9LD6). However, two independent pieces of work providing a similar description of the function of tRNA-Gly-derived small RNA lend support to the possibility of tsRNA as a potential target of HCC.

Other than the promotion of migration, we first identified that 5′tRF-Gly promotes HCC cell proliferation by regulating CEACAM1 and determined the function of CEACAM1 in HCC cells. In our clinical sample analysis, the high 5′tRF-Gly expression group (fold change > 1.5) had a significantly larger tumor size and higher tumor metastasis ratio in HCC patients. It is worth noting that a borderline significant correlation was observed for the high 5′tRF-Gly expression group and poorly differentiated tumor (Table 1, $p = 0.076$). The lower the degree of tumor differentiation, the closer the cell function to LCSCs. Therefore, the results led us to speculate whether 5′tRF-Gly is related to LCSC. Interestingly, Zhou et al. observed that Gly-tRF mimics increased the LCSC subpopulation proportion. Perhaps the 5′tRF-Gly reported here also has a regulatory effect on LCSC through a certain pathway, and 5′tRF-Gly overexpression could make more HCC cells stay at the poorly differentiated level, but our study did not explore this in depth. Such research could be the key to early intervention in poorly differentiated HCC, improving the prognosis.

Interestingly, when we detected the expression of EMT markers, we only found the promoting effect of CEACAM1 on E-cadherin but did not find its regulatory effect on N-cadherin. In HCC progression, EMT plays a vital role in the early stages of tumor metastasis. The reduction of E-cadherin leads to decreased cell–cell adhesion, allowing cancer cells to spread to the surrounding or distant tissues, causing tumor metastasis.[32] A large body of evidence shows that hepatocellular EMT is a dedifferentiation of malignant hepatocytes, hepatic progenitor cells, or hepatic stellate cells.[33] This could explain why we found that the high 5′tRF-Gly expression group has a higher proportion of poorly differentiated tumors during clinical data analysis.

Carcinoembryonic antigen-related cell adhesion molecule 1 is a transmembrane glycoprotein and a member of the CEA family of highly glycosylated cellular adhesion molecules.[34] Under normal insulinemic conditions, CEACAM1 mediates insulin sensitivity and restricts lipogenesis in the liver by promoting appropriate insulin clearance.[35,36] The severity of NAFLD was found to be linearly correlated with reduced levels of hepatic CEACAM1.[37] Nonalcoholic steatohepatitis is a progressive form of NAFLD and can induce the progression of liver fibrosis and cirrhosis, eventually developing into HCC.[36] Our experimental results showed that the silencing of CEACAM1 can promote the proliferation and migration of HCC cells. Horst and Wagener[24] proved CEACAM1 is downregulated compared to healthy tissue specimens in HCC. According to the survival curves in the Kaplan–Meier Plotter database, higher expression of CEACAM1 leads to longer PFS in HCC, and a borderline significant correlation was found in the GEPIA2 database between high expression of CEACAM1 and longer overall survival time (Figure S1E, $p = 0.1$). Coupled with our experimental results, we have reason to believe that CEACAM1 inhibits the progression of HCC by downregulating MMP2 and cyclin D1 and upregulating E-cadherin. Combined with the role of CEACAM1 in NAFLD, we speculate that the absence of CEACAM1 could promote the development of HCC. However, the regulation mechanism of the expression of these genes by CEACAM1 remains to be studied. In contrast, related studies have reported that the increased expression of CEACAM1 in LCSC can resist natural killer cell-mediated cytotoxicity.[38] Additionally, the long cytoplasmic domain isoform of CEACAM1 is associated with invasion and recurrence of HCC.[39] This suggests its complex role and duality in liver cancer, and further research is needed to reveal its function.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (No. 81773096, 82072650, and 81902405), Key Research and Development Program of Zhejiang Province (No. 2018C03085 and 2021C03121), 2019 Liver Cancer Diagnosis and Treatment Communication Fund (CXPJJH11900009-12), and the Health Commission of Zhejiang Province (JBZX-202004).

DISCLOSURE

The authors declare no conflict of interest.

ETHICS STATEMENT

Ethics approval for this study was obtained from the Ethics Committee of the Second Affiliated Hospital of Zhejiang University School of Medicine (#2021–0596, 2021 updated).

All participants gave their informed consents for specimen and clinical information collection.

Registry and registration no. of the study: N/A.

All animal protocols were approved by Institutional Animal Care and Use Committee at the Second Affiliated Hospital of Zhejiang University School of Medicine (#2021–169, 2021 updated).

ORCID

Weilin Wang https://orcid.org/0000-0001-9432-2649

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How to cite this article: Liu D, Wu C, Wang J, et al. Transfer RNA-derived fragment 5′tRF-Gly promotes the development of hepatocellular carcinoma by direct targeting of carcinoembryonic antigen-related cell adhesion molecule 1. *Cancer Sci*. 2022;113:3476-3488. doi: 10.1111/cas.15505