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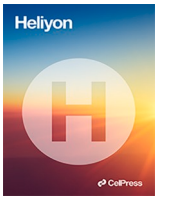

Research article

LRRC59 promotes the progression of oral squamous cell carcinoma by interacting with SRP pathway components and enhancing the secretion of CKAP4-containing exosomes

Qijun Sun [a,1], Lili Jin [b,1], Shunli Dong [b], Ling Zhang [a,*]

[a] Department of Stomatology, Huzhou Central Hospital, Affiliated Central Hospital Huzhou University, Huzhou, 313000, Zhejiang, China
[b] Huzhou Key Laboratory of Molecular Medicine, Huzhou Central Hospital, Affiliated Central Hospital Huzhou University, Huzhou, 313000, Zhejiang, China

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ABSTRACT

Background: As a ribosome receptor, LRRC59 was thought to regulate mRNA translation on the ER membrane. Evidence suggests that LRRC59 is overexpressed in a number of human malignancies and is associated with poor prognoses, but its primary biological function in the development of oral squamous cell carcinoma (OSCC) remains obscure.

Objective: The purpose of this study is to investigate at the expression changes and functional role of LRRC59 in OSCC.

Methods: LRRC59 gene expression and correlation with prognosis of OSCC patients were first examined using the data from The Cancer Genome Atlas (TCGA) databases. Following that, a series of functional experiments, including cell counting kit-8, cell cycle analysis, wound healing assays, and transwell assays, were carried out to analyze the biological roles of LRRC59 in tumor cells. Mechanistically, we employed Tandem Affinity Purification-Mass Spectrometry (TAP-MS) approach to isolate and identify protein complexes of LRRC59. Downstream regulatory proteins of LRRC59 were verified through immunoprecipitation and immunofluorescence experiments. Furthermore, we isolated exosomes from OSCC cell supernatant and conducted co-culture experiments to examine the effect of LRRC59 knockdown on OSCC cells.

Results: In samples from OSCC patients, LRRC59 was highly expressed and correlated with poor prognoses. Moreover, the gene sets analysis based on TCGA RNA-seq data indicated that LRRC59 seemed to be strongly related with protein secretory and OSCC migration. Upregulated levels of LRRC59 are more prone to lymph node metastasis in OSCC patients. LRRC59 knockdown impaired the ability of OSCC cell proliferation, migration, and invasion invitro. Mechanistically, our TAP-MS data situate LRRC59 in a functional nexus for mRNA translation regulation via interactions with SRP pathway components, translational initiation factors, CRD-mediated mRNA stabilization factors. More importantly, we found that LRRC59 interacted with cytoskeleton-associated protein 4 (CKAP4) and promoted the formation of CKAP4-containing exosomes. We also revealed that the LRRC59-CKAP4 axis was a crucial regulator of CKAP4-containing exosome secretion in OSCC cells for migration and invasion.

* Corresponding author. Department of Stomatology, Huzhou Central Hospital, Affiliated Central Hospital Huzhou University, No. 1558, Sanhuan North Road, Huzhou, 313000, Zhejiang Province, China.
E-mail address: zlu@hzhospital.com (L. Zhang).
[1] These authors contributed equally to this work.

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Conclusions: Therefore, based on our findings, LRRC59 may serve as a potential biomarker for OSCC patients, and LRRC59-induced exosome secretion via the CKAP4 axis may serve as a potential therapeutic target for OSCC.

1. Introduction

Oral squamous cell carcinoma, a locally aggressive malignancy, contributes for more than 90% of cases of oral cancer [1]. Despite advancements in surgery and various treatments, the 5-year survival rate for patients with OSCC is still appalling [2]. As of now, little is known about the components and molecular mechanisms that contribute to the genesis of OSCC tumors. Therefore, gaining a deeper comprehension of the molecular basis of OSCC may open approaches that improve OSCC patients' prognoses (see Table 1).

Cancer patients frequently experience translational dysregulation spurred on by overactive upstream signaling pathways and/or alterations to the expression or function of translational machinery components [3]. The primary site to produce secretory, membrane, and organelle proteins in eukaryotic cells is the endoplasmic reticulum (ER) [4]. Type II transmembrane protein LRRC59 has four LRRs and a coiled-coil domain on its extracellular side and a short C-terminal region that faces the ER lumen on its cytoplasmic side [5]. Emerging studies have shown that LRRC59, as a ribosome receptor, was to regulate mRNA translation on the ER membrane [6]. In various cancer types, increased levels of LRRC59 mRNA are linked to adverse clinical outcomes, including lung adenocarcinoma [7], urothelial carcinoma [8] , breast cancer [9]. However, the significance of LRRC59 signaling in OSCC development is not fully understood. Therefore, the purpose of this work is to examine the expression alterations and functional significance of LRRC59 in OSCC.

2. Materials and methods

2.1. Analyze LRRC59 expression in human cancers

The UALCAN database (http://ualcan.path.uab.edu) were used to compare LRRC59 expression between head and neck squamous cell carcinoma and paired normal tissue, it integrated RNA-sequencing and clinical data from the TCGA and offered a platform for analyzing gene expression of tumor and normal tissues.

2.2. RNA-sequencing data from TCGA

The TCGA's HNSCC programs (https://genome-cancer.ucsc.edu/) provided access to gene expression data and clinical information for OSCC patients (332 cases, Workflow type: HTseq-FPKM). The analysis did not include samples from the hypopharynx, larynx, oral cavity, or tonsil. Missing values were defined as clinical features that were unavailable or unknown.

2.3. Tissue microarray (TMA)-based immunohistochemistry assays

Tissue microarray chips were purchased from Outdo Biotech Company; they contained 55 samples of OSCC and 5 samples of

Table 1
Correlation of the expression of LRRC59 with clinicopathologic features in squamous cell carcinoma.

Variables	N (%)	LRRC59[a]		p-value
		High	Low	
Gender				0.7033
Male	27(50%)	17	10	
Female	27(50%)	18	9	
Age(years)				0.9452
<55	15(28%)	8	7	
≥55	38(72%)	27	11	
Tumor size(cm)				0.0473*
≥3	31(69%)	25	6	
<3	14(31%)	7	7	
Lymph node metastasis				0.0133*
NO	22(74%)	12	10	
Yes	8(26%)	8	0	
TNM stage				0.0092**
I+II	42(82%)	26	16	
III+IV	9(18%)	9	0	
Pathological grade				0.0164*
I+II	41(80%)	25	16	
III+IV	10(20%)	10	0	

*P < 0.05, **P < 0.01 was considered significant (Chi-square test between 2 groups).
[a] Fold change (FC) (tumor tissues relative to normal tissues) is greater than or equal to 2.0 for high expression, and less than 2.0 for low expression.

normal control (Shanghai, China). Taizhou Hospital (Zhejiang, China) provided the samples for this study. Detailed information of clinicopathological features for these patients were displayed in Table S1.

IHC studies of LRRC59 were performed in tissue microarray as below: following deparaffinization and drying, Rabbit polyclonal LRRC59 antibody was applied to the tissue microarray chips overnight at 4 °C (dilution 1:500, Abcam) shortly after epitope retrieval, hydrogen peroxide treatment, and blocking of non-specific antigens. A secondary antibody was then applied to the chips, and the signal was detected using a DAB staining kit. All stainings were assessed based on the strength and extensity of positive cells, with the intensity (0 = genitive, 1 = weak, 2 = moderate, and 3 = strong) and extensity (0 = 10% or less of cells stained positive; 1 = 11%–40%; 2 = 41%–70%; 3 = 71% or more) of tumor staining being examined. To obtain the final score, the total extensity score was multiplied by the highest intensity score (maximum value of 9).

2.4. Cell culture

The human OSCC cell line CAL27 was obtained from Shanghai Zhong Qiao Xin Zhou Biotechnology. The CAL27 cells were grown in DMEM media from Gibco in the United States, which contains 10% fetal bovine serum (FBS, Corning), and the cells were passaged once they had reached confluence using a 0.25 percent trypsin-EDTA solution. The cells were maintained at 37 °C in humidified air with 5% CO2.

2.5. Lentivirus production and cell infection

LRRC59 gene expression was knocked down using the short hairpin RNA (shRNA) lentivirus transduction method. Genepharma Shanghai provided the lentivirus vector and two short hairpin RNA (shRNA) targeting LRRC59. The following LRRC59 sequences were chosen as targets: shRNA#1:5-CCTGGATCTGTCTTGTAATAA-3; shRNA#2:5-GCAGTGTAAAGCAGTGTGCAAA-3; shCon:5'-AATACGGCGATGTGTCAGG-3', shRNAs were packaged into lentiviruses by cotransfecting 293T cells using the packaging plasmids pMD2G and pSPAX2. After 48 h of transfection, the supernatant was collected and used to infect CAL27 cells. For two days, infected cells were selected in puromycin (MCE 2 μg/mL)-containing medium.

For SFB-tagged LRRC59 stably express in CAL27 cell lines, LRRC59-containing pDONR201 derivative constructs were introduced into the Gateway-compatible lentiviral vector. CAL27 cells were infected with viral supernatants 48 h after cotransfecting lentiviral vectors and packaging plasmids into HEK293T cells. and Stable pools were selected using 2 μg/ml puromycin.

2.6. Retrovirus production and infection

After transferring LRRC59-containing pDONR201-derived constructs into the Gateway-compatible pEF1A-HA-Flag retroviral vector, LRRC59-containing retroviral vectors, pCL-Eco, and VSV-G were cotransfected into HEK293T cells, and the virus supernatant was harvested. CAL27 cells were infected with viral supernatant and selected in growth medium containing 2 μg/ml puromycin (MCE).

2.7. Western blot

NETN lysis buffer was used to homogenize cells for western blotting. They were boiled for 15 min with 2 × lysis loading buffer. Supernatants were added into SDS-PAGE gels. Primary antibodies were incubated with nitrocellulose membranes at 4 °C for overnight before being reacted with HRP-conjugated secondary antibodies and analyzed.

2.8. CCK-8 assay

CAL27 cells infected with lentiviruses were grown to the logarithmic phase in culture. The cells were then reseeded in triplicate into 96-well plates at a density of 2,000 cells per well. Cells were treated with 10 μl of Cell Counting Kit-8 (CCK-8, Beyotime, China) per well and incubated for 2 h after initial incubation for 12, 24, 48, and 96h. The absorbance was then measured at 450 nm using a microplate reader.

2.9. Cell-cycle analysis

Lentiviral-infected CAL27 cells were cultured for 96 h. After collecting the cells, they were fixed in cold 75% ethanol for 12 h. Cells were treated with PI buffer following a PBS wash (C6031, US Everbright Inc) for 20 min at 4 °C. The cell cycle state was then determined using the FACS Calibur II (Becton-Dickinson, USA).

2.10. Immunofluorescence (IF)

Cells were fixed with PBS containing 4 percent PFA before being permeabilized for 6 min in PBS containing 0.5 percent Triton X-100 for immunofluorescent labeling. The cells were first blocked in PBS containing 5% bovine serum albumin, followed by an overnight or 1-h incubation with antibodies diluted in PBS, three PBS washes, and a 1-h incubation with a secondary antibody (conjugated to Alexa Fluor 488, 555; CST). The cells were washed, then counterstained with DAPI, then coated with PBS that contains 50% glycerol.

2.11. Transwell assay

CAL27 cells infected with lentiviral were grown to logarithmic phase. Cal27 cells were obtained, washed in PBS, and then resuspended in serum-free medium at a concentration of 106 cells/ml. Following that, 1.5105 cells were placed in Transwell inserts, and 10% FBS DMEM medium was poured into the 24-well plate's bottom(Corning™ 3464, Corning, NY). After 24 h of incubation, Cotton swabs were used to remove cells from the upper surface of the membranes. Crystal violet was used to dye the cells for 15 min after they had been fixed in 4% PFA underneath the membranes. Using an inverted microscope, three randomly selected fields from each group were recorded.

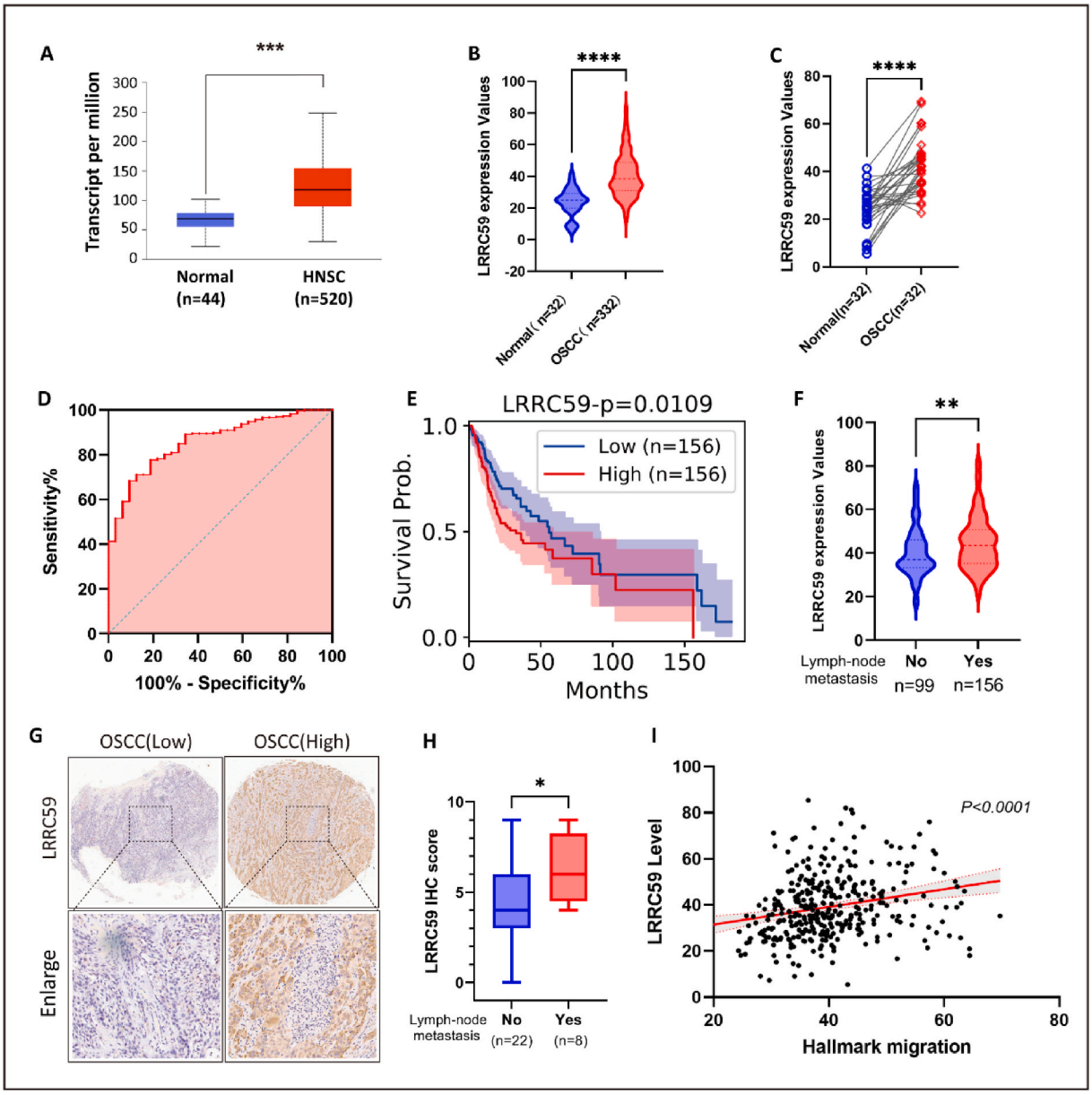

Fig. 1. The high expression of LRRC59 is associated with OSCC malignancy. (A) LRRC59 expression of normal samples and tumor samples in TCGA-HNSCC database. (B) LRRC59 expression of normal samples and tumor samples in OSCC patients from TCGA-HNSCC database. (C) LRRC59 expression of tumor tissues and paracancer tissues in OSCC patients from TCGA-HNSCC database. (D) ROC analysis of LRRC59 expression showing promising discrimination power between tumor and normal tissues. (E) Kaplan-Meier curves of OS with different expression level of LRRC59. (F) LRRC59 level in non-lymph-node metastasis (No) and lymph-node metastasis (Yes) of TCGA OSCC tissues. (G) Representative IHC staining images of LRRC59 level in OSCC tissues. (H) LRRC59 level of nonlymph-node metastasis (No) and lymph-node metastasis (Yes) in OSCC tissues. (I) Association of LRRC59 level with hallmark migration based TCGA OSCC data. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

2.12. Tandem affinity purification (TAP)

Twenty 10 cm dishes of CAL27 cells that stably express SFB-tagged were lysed with NETT buffer (25 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA, 1 % Triton X-100) containing protease inhibitors (proteinase inhibitor cocktail, roche) at 4 °C for 30 min, and sonicated for 15s to extract chromatin-bound proteins. The supernatants were centrifuged at 15,000×*g* for 10 min to remove debris before being incubated for 3 h at 4 °C with gentle rocking in the presence of 100 μl of streptavidin-conjugated beads. (GE Healthcare). Before being eluted for 2 h at 4 °C with 1 mg/ml biotin, the immunocomplexes were washed three times using NETN buffer (20 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA, and 1 % NP-40) (Sigma-Aldrich). Next, 60 μl of S-protein Agarose beads were added to the eluates and incubated at 4 °C for 3 h with the eluates (EMD Millipore). NETN buffer was used to wash the proteins three times before SDS-PAGE separation and mass spectrometry analysis.

2.13. Construction and analysis of PPI network

The protein-protein interaction (PPI) network was constructed using STRING (https://string-db.org/), with minimum required interaction score set in 0.4. The list of genes' gene IDs was entered into STRING, and biological classification, including GO term enrichment analysis, KEGG pathways, and Reactome pathways enrichment analysis, were assessed for the list of genes.

2.14. Immunoprecipitation

NETN lysis solution that has protease inhibitors added was used to lyse the cells. The soluble fractions were obtained after centrifugation was used to remove cell debris. The lysates were incubated with 30 μl S-protein Agarose beads for 2 h at 4 °C. To detect endogenous interaction between CKAP4 and LRRC59, the lysates were incubated with 1.5 μg CKAP4 antibody and 25 μl of a 50% slurry of protein A-Sepharose beads (GE Healthcare) for 2 h at 4 °C. The precipitates were probed with the designated antibodies after being washed three times with NETN buffer.

2.15. Exosome isolation

Exosome-depleted FBS (DMEM) containing 10% was used to culture CAL27 cells. After 48 h, After 15 min of centrifugation at 10,000g, the medium was collected in an ultrafiltration tube (100kd). Following the manufacturer's protocol, we isolated exosomes from the medium using the AVIDTM Exosome Isolation Regent (Aivd Biotech, Shenzhen, China). For further functional tests or protein detection, the exosome pellets were resuspended in Regent C and filtered (0.22 μm pore size; Millipore). A transmission electron microscope (TECNAI G2 Spirit Biotwin) operating at 120 kV was used to view the exosomes. Purified exosomes were used in in vitro tests at a concentration of 20 μg/ml.

2.16. Statistical analysis

The assessments of the Gene Ontology and KEGG pathway enrichment were conducted using the Database for Annotation, Visualization and Integrated Discovery (STRING) bioinformatics resource. P values < 0.05 were regarded as statistically significant when using the GraphPad Prism (version 9.0, GraphPad Software Inc., CA) for statistical analysis. The data were given as the mean ± standard deviation.

3. Results

3.1. Elevated LRRC59 expression is associated with malignancy of OSCC

Oral squamous cell carcinoma is the most prevalent histologic type of head and neck squamous cell cancer. (HNSCC) [10]. We first analyzed LRRC59 expression of HNSCC and non-cancerous tissues by the UALCAN website (based on TCGA database). The results showed that LRRC59 was significantly elevated in HNSCC (Fig. 1A). To assess the expression of LRRC59 in OSCC, a total of 332 OSCC patients were obtained after excluding non-oral samples. The results indicated that LRRC59 expression was substantially higher in tumor tissues than in non-tumor tissues. (Fig. 1B). We subsequently compared LRRC59 expression in tumor tissues to that of its paracrine counterparts and found that LRRC59 was significantly elevated in OSCC samples (Fig. 1C). Besides, LRRC59 can be employed as a marker to identify tumor cells from non-tumor cells, according to the receiver operating characteristic analysis (Fig. 1D). In addition, OSCC patients with high LRRC59 expression had a significantly lower long-term overall survival rate (Fig. 1E).

Regional lymph node metastasis is well-known to have a poor prognostic impact on OSCC [11]. We first evaluated the association between LRRC59 expression and the lymph node metastasis of OSCC patients based on TCGA data. As shown in Fig. 1F, LRRC59 overexpression was significantly correlated with the lymph node metastasis. The gene sets analysis based on TCGA RNA-seq data of OSCC patients also showed that LRRC59 expression highly associated with OSCC cells migration (Fig. 1I). Additionally, immunohistochemical analysis in a tissue microarray that included 55 OSCC and 5 noncancerous adjacent oral tissues further confirmed the remarkable correlation of LRRC59 expression and lymph node metastasis (Fig. 1G and H). The current data indicate that up-regulated LRRC59 is significantly associated with OSCC malignancy, suggesting that it may act as a potential biomarker for progression and metastasis in OSCC.

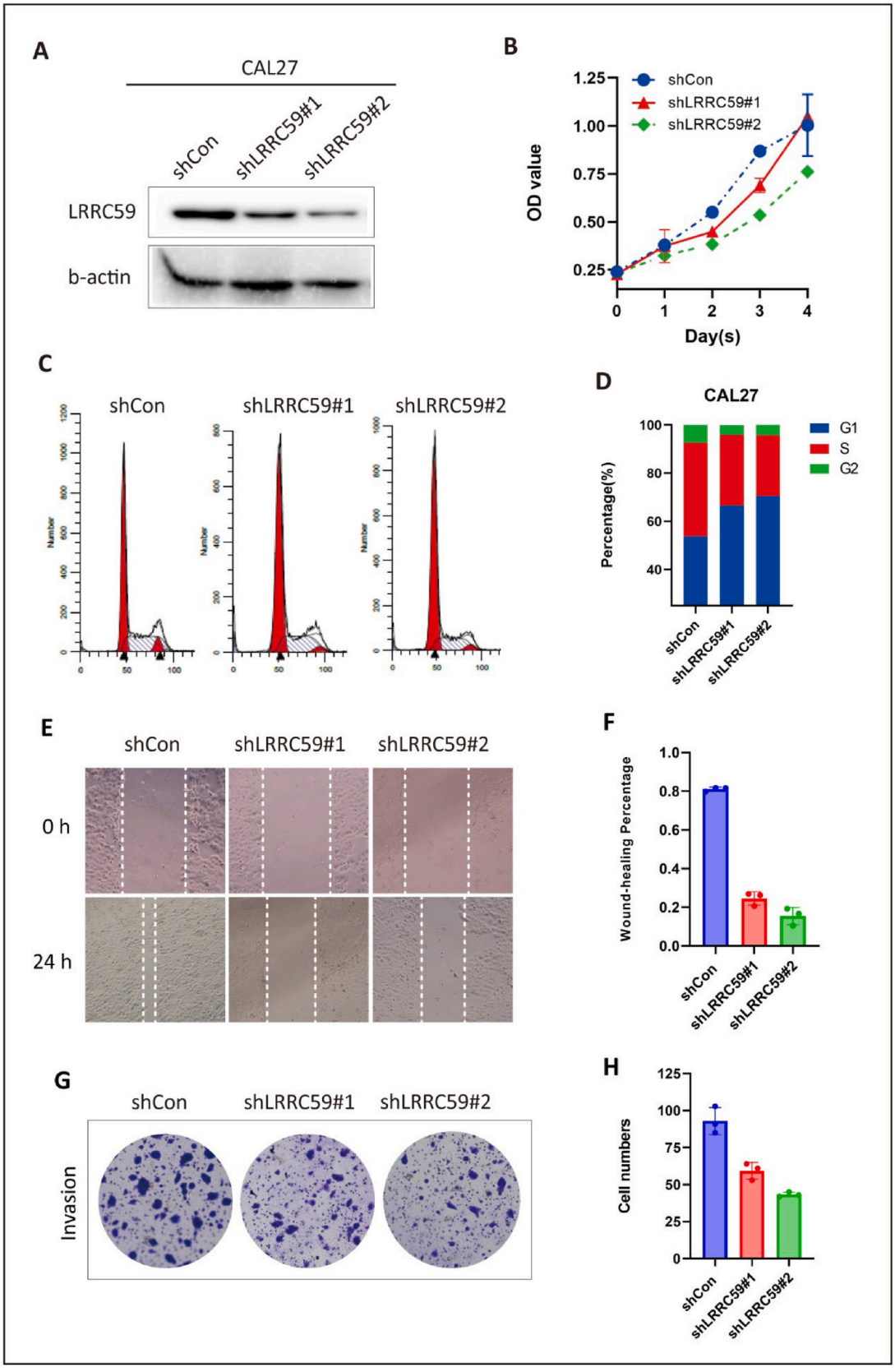

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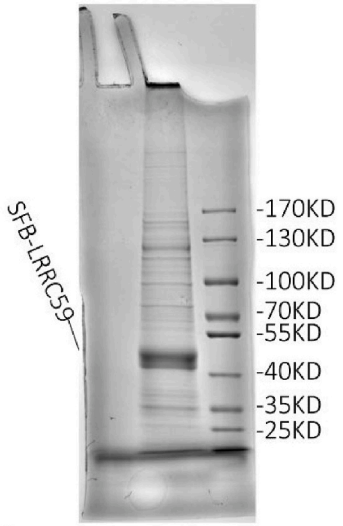
Fig. 2. Knockdown of LRRC59 decreased OSCC cell proliferation and migration in vitro. (A) Western blotting analysis on LRRC59 expression following shRNA-mediated knockdown in CAL27 cells. (B) shRNA knockdown of LRRC59 in CAL27 cells significantly inhibited cell proliferation in CCK8 assay. (C, D) Silencing LRRC59 led to a significant G1 arrest with a parallel S and G2/M-phase reduction. (E, F) The silencing of LRRC59 reduced CAL27 cell migration in the Wound healing assay. (G, H) The depletion of LRRC59 decreased CAL27 cell invasion in the Transwell assay.

3.2. Knockdown of LRRC59 Inhibits OSCC cell proliferation, migration and invasion

The clinical pathological findings revealed that LRRC59 overexpression may be involved in the progression of OSCC. Therefore, we conducted functional experiments in vitro with oral carcinoma cell line CAL27 to further investigate its role. CAL27 cells were infected

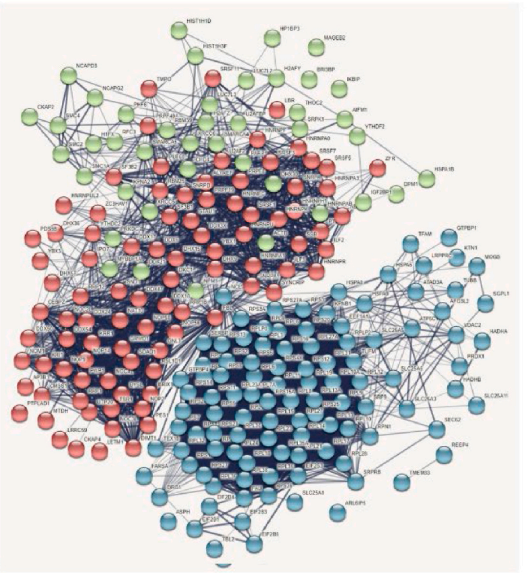
A



B

Gene ID	Peptides	Coverage
LRRC59	30	66.78
HNRNPU	33	42.91
RPS4X	23	69.96
DDX21	29	44.32
* CKAP4	24	56.98
XRCC5	34	46.99
RPS3A	17	58.33
PARP1	30	35.50
XRCC6	27	44.17
RPL4	23	43.56
RPS2	13	43.69
DHX9	31	27.56
RPS3	21	75.72
YBX1	7	46.60
RPL3	16	40.69
RPL7A	18	45.11
GNL3	19	39.71
RPS7	13	59.28
RPS6	9	34.94
DDX5	13	32.25
HSPA5	19	38.99
NPM1	8	36.39

C



D

Biological Process (Gene Ontology)

GO-term	description	count in network	strength	false discovery rate
GO:0070934	CRD-mediated mRNA stabilization	5 of 5	1.79	1.83e-05
GO:0006614	SRP-dependent cotranslational protein targeting to membra...	71 of 96	1.66	9.04e-81
GO:0006613	Cotranslational protein targeting to membrane	73 of 100	1.65	1.02e-82
GO:0045047	Protein targeting to er	75 of 110	1.62	1.99e-83
GO:0019083	Viral transcription	70 of 115	1.57	2.57e-75
GO:0000184	Nuclear-transcribed mrna catabolic process, nonsense-med...	70 of 119	1.56	1.37e-74
GO:1904044	Response to aldosterone	3 of 5	1.56	0.0101
GO:1902255	Positive regulation of intrinsic apoptotic signaling pathway ...	3 of 5	1.56	0.0101
GO:1903608	Protein localization to cytoplasmic stress granule	4 of 7	1.54	0.0011
GO:0006413	Translational initiation	78 of 141	1.53	7.47e-82

KEGG Pathways

pathway	description	count in network	strength	false discovery rate
hsa03010	Ribosome	68 of 130	1.5	4.65e-70
hsa03060	Protein export	8 of 23	1.33	2.64e-06
hsa03040	Spliceosome	33 of 132	1.18	1.77e-24
hsa03008	Ribosome biogenesis in eukaryotes	9 of 77	0.85	0.00067
hsa03013	RNA transport	14 of 160	0.73	7.65e-05
hsa04141	Protein processing in endoplasmic reticulum	12 of 165	0.65	0.0017

Fig. 3. The Interactome of LRRC59 in OSCC Cells. (A, B) Cal27 cells stably expressing SFB-tagged-LRRC59 were used for TAP of protein complexes specifically from whole cell lysate and determined by Coomassie blue staining. Tables are summaries of proteins identified by mass spectrometry analysis. (C, D) Biological processes enrichment analysis of LRRC59 interactome and its functional partners (STRING). The protein-protein interaction (PPI) network was constructed using STRING (https://string-db.org/), Biological classification was evaluated for the list of genes including GO term enrichment analysis, KEGG pathways and Reactome pathways enrichment analysis.

with shCon or LRRC59-shRNA-expressing lentiviruses, and LRRC59 shRNA knockdown efficiency was assessed using western blots (Fig. 2A). CCK8 assay indicated that the proliferation of LRRC59-knockdown cells was significantly inhibited (Fig. 2B). Secondly, flow cytometric analysis of cell cycle distribution showed that silencing LRRC59 led to a significant G1 arrest with a parallel S and G2/M-phase reduction (Fig. 2C and D). These results indicate that LRRC59 knockdown impaired cell proliferation.

The migration and invasion abilities of OSCC cells were then assessed using wound healing migration and transwell invasion assays. In the wound healing migration assay, the shCon group cells' wound area considerably shrunk after 24 h (Fig. 2E and F). In contrast, after 24 h, the gap persisted in LRRC59-knockdown cells. The transwell assay showed that the number of invasive cells were

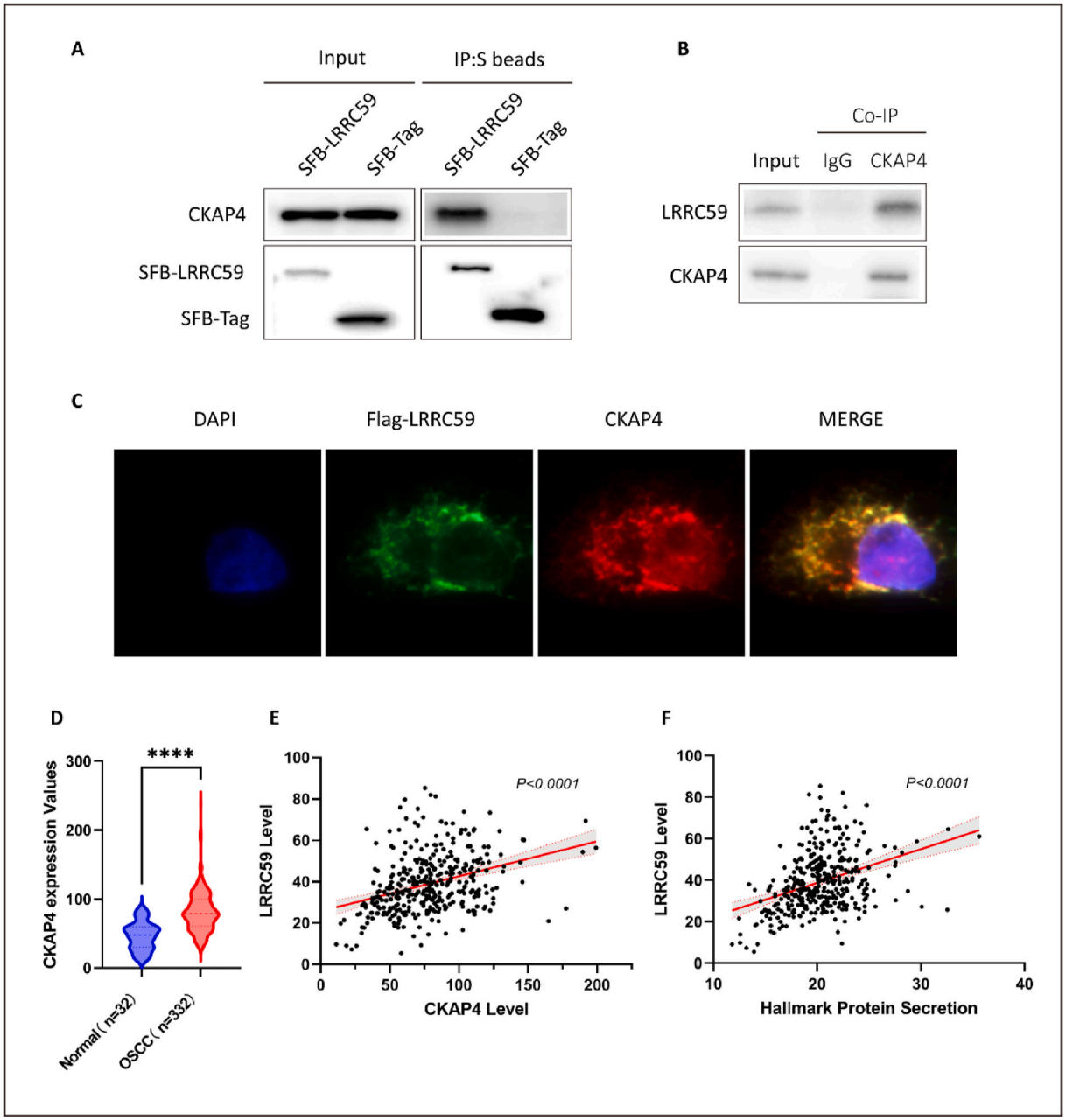


Fig. 4. LRRC59 Interacted with CKAP4. (A) Co-IP analysis of CAL27 cells transfected with the SFB-Vector and SFB-LRRC59, Cell lysates were immunoprecipitated with S-protein Agarose beads and western blot analysis was performed as indicated. (B) Association of endogenous LRRC59 with the CKAP4 in Cal27 cells was performed by co-immunoprecipitation using anti-CKAP4 antibody. (C) Colocalization analysis of LRRC59 and CKAP4 in Flag-LRRC59 stably expressing CAL27 cells with antiCKAP4 antibody and anti-Flag antibody. (D) The expression of CKAP4 in in normal tissues and OSCC tissues obtained from the TCGA. (E) Correlation analysis of the expression level of LRRC59 and CKAP4. (F) Association of LRRC59 level with hallmark protein secretion based TCGA OSCC data. ****p <0.0001.

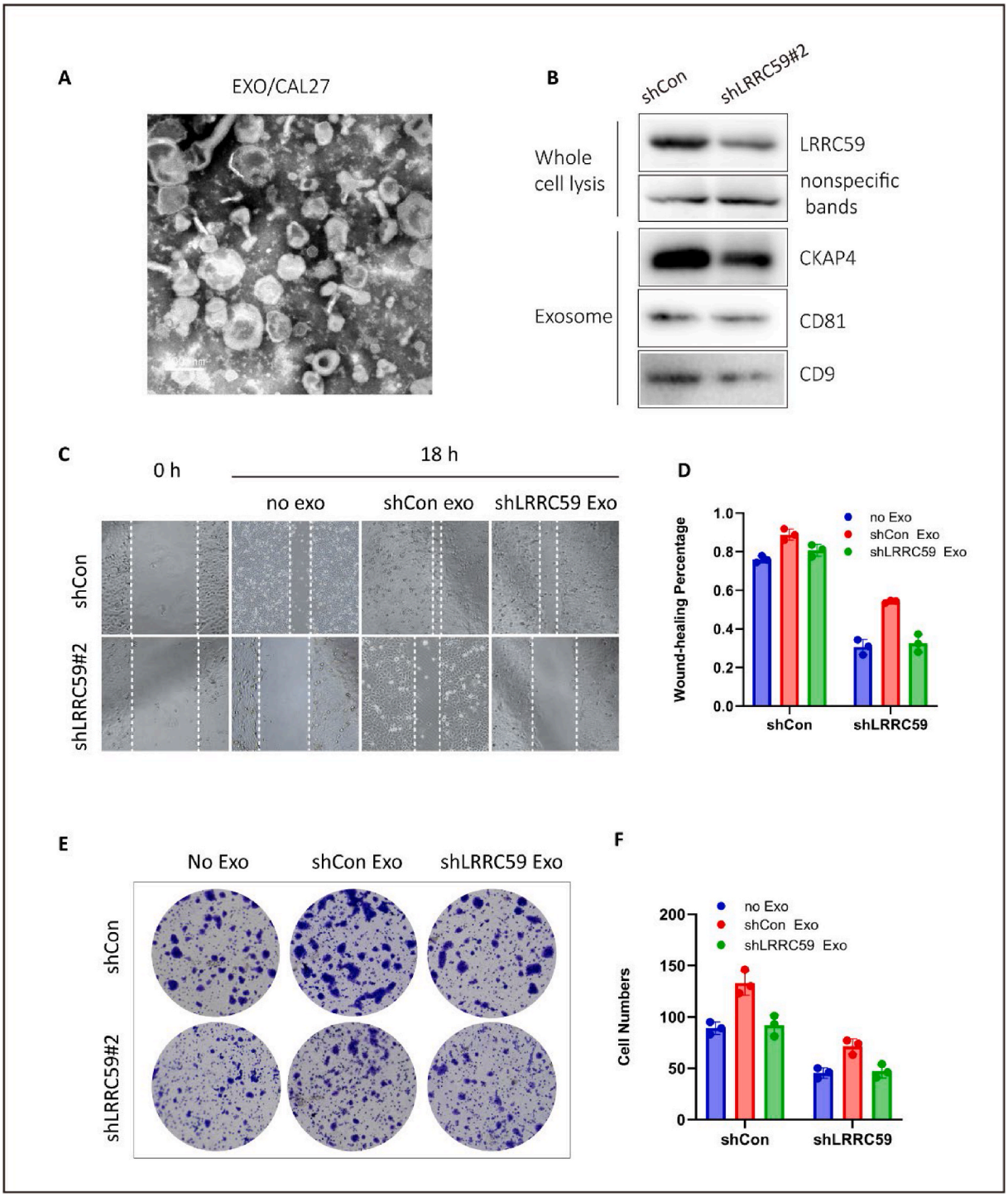


Fig. 5. LRRC59 enhances the production of CKAP4-containing exosomes to promote OSCC cell migration and invasion. (A) Representative electron micrograph of exosomes isolated from Cal27 (scale bar, 100 nm). (B) Western blot analysis of CD9, CD81 and CKAP4 levels in exosomes of LRRC59-depleted and shRNA control Cal27 cells. (C) Wound healing assay of shRNA control and shLRRC59 cells, as well as the effect of exosomes secreted from LRRC59-depleted and shRNA control Cal27 cells (mean ± SEM, n = 3 independent experiments). (D) Trans-well assay of shRNA control and shLRRC59 cells, as well as the effect of exosomes secreted from LRRC59-depleted and shRNA control Cal27 cells.

significantly decreased in LRRC59-knockdown culture (Fig. 2G and H). These results indicate that LRRC59 knockdown impaired cell migration and invasion.

3.3. Mapping the LRRC59-Associated protein network by TAP-MS

To further elucidate the molecular mechanisms of LRRC59 in OSCC, tandem affinity purification and mass spectrometry (TAP-MS) approach was used to identify LRRC59-interacting proteins in CAL27 cells. The CAL27 cells stably expressing LRRC59 fused with a C-terminal SFB triple tags were generated. After confirming the expression of the bait protein, TAP was performed on the LRRC59 stable cells. The isolated proteins were made visible using a Coomassie blue staining procedure (Fig. 3A). The associated proteins with LRRC59 were further examined using mass spectrometry (MS) (Fig. 3B). Each detected hit was given a SEQUEST HT score, and any interaction with a SEQUEST HT score of 1 and Unique Peptides of 2 was designated a high-confidence interacting protein. Through this filtration, our analysis identified 320 LRRC59 interacting proteins.

Next, the functional enrichment analysis was performed by STRING (Fig. 3C and D). KEGG pathway analysis showed that abundant in the LRRC59 interactome were subunits of the ribosome, spliceosome, protein export. Gene ontology (GO) term analysis showed LRRC59 interactome enrichment in multiple signaling pathways, mainly involved in signal recognition particle (SRP)-dependent pathway, translational initiation, nuclear-transcribed mRNA catabolic process, CRD-mediated mRNA stabilization. As a ribonucleoprotein complex, SRP is crucial for the co-translational targeting of proteins to appropriate membranes and secretory pathways [12]. These interactions link LRRC59 to the regulation of ER-based secretory and membrane protein production in OSCC progression.

3.4. LRRC59 interacted with CKAP4

Interestingly, we found that CKAP4, an endoplasmic reticulum (ER) protein, as major LRRC59-associated proteins (Fig. 3B). Recently, it has been reported that CKAP4 is a biomarker in exosomes and CKAP4-Bearing Exosomes promote metastasis in several kinds of cancers [13–15]. Co-IP analysis of CAL27 cells transfected with the SFB-Vector or SFB-LRRC59 revealed that LRRC59 binds CKAP4 strongly (Fig. 4A). We further validated the interaction of CKAP4 with LRRC59 at endogenous levels (Fig. 4B). We also examined the co-localization of LRRC59 and ckap4 in Flag-LRRC59 express CAL27 stably cells. In fluorescence immunocytochemistry experiments, LRRC59 and CKAP4 were co-localized in the cytoplasm (Fig. 4C). Additionally, we examined the expression of CKAP4 in normal tissues (n = 32) and OSCC tissues (n = 332) obtained from the TCGA. We found that CKAP4 was significantly upregulated in OSCC tissues when compared to the surrounding nontumor tissues (Fig. 4D). The expression of LRRC59 and CKAP4 in OSCC tissues showed a positive correlation, according to Pearson correlation analysis (Fig. 4E). Furthermore, the gene sets analysis based on TCGA data of OSCC patients showed in Fig. 4F, LRRC59 seemed to be closely related to protein secretion in OSCC. These results imply that the interaction of LRRC59 and CKAP4 may be critical for CKAP4-containing exosomes secretion in the progression of OSCC.

3.5. LRRC59 increases CKAP4 levels in exosomes to promote cell migration and invasion

Next, we examine whether LRRC59 was essential to stimulate the generation of exosomes that contained CKAP4. Exosomes were isolated from LRRC59-shRNA and shCon CAL27 cells by the AVID ™ Exosome Isolation kit, and The structural characteristics of exosomes were confirmed using phase contrast electron microscopy. (Fig. 5A). Western blot analysis verified the expression of the exosome markers CD9 and CD81. (Fig. 5B). The results showed that exosomes secreted by CAL27 shCon cells enriched in CKAP4 protein, and knockdown LRRC59 significantly decreased CKAP4 level in exosomes. Moreover, LRRC59-depleted cells showed a slower rate of wound healing as compared to Cal27 Control group cells, the healing rate of Cal27 LRRC59-shRNA and shCon cells was mitigated significantly after treated with exosomes secreted from shCon CAL27 cells but not LRRC59-depletion exosomes (Fig. 5C and D). Additionally, the enhance of cell invasion ability treated with exosomes secreted from OSCC cells also been observed in transwell invasion experiments (Fig. 5E and F). Our research thus demonstrated that LRRC59 enhances the production of CKAP4-containing exosomes to promote OSCC cell migration and invasion.

4. Discussion

Due to its high level of heterogeneity and complex aetiologic factors, the prognosis of patients with OSCC is challenging to predict or describe [16]. In current study, we found elevated LRRC59 expression in OSCC tissues, which was strongly associated with OSCC patients' poor prognoses. Moreover, the gene sets analysis based on TCGA data of OSCC patients showed that LRRC59 seemed to be considered in relation to protein secretion and OSCC migration. Our findings further indicate that OSCC patients with higher LRRC59 expression in tissue microarray chips are more probable to lymph node metastasis. LRRC59 knockdown impaired the ability of OSCC cell proliferation, migration, and invasion invitro. These results demonstrate that elevated LRRC59 expression is significantly correlated with OSCC malignancy and that it may serve as a potential biomarker for OSCC progression and metastasis.

It is well documented that elevated endoplasmic reticulum activity is necessary to support the synthesis, folding, and modification of secretory and membrane proteins for cancer cell growth and survival [17]. As a ribosome receptor, LRRC59 is believed to regulate the abundance of overall ribosome on the ER [18,19]. Additionally, LRRC59 regulates mRNA translation directly by interacting with translation elements and SRP apparatus on ER [20]. Consistently, via tandem affinity purification-mass spectrometry, we found that the LRRC59 interactome was dominated by SRP pathway components. SRP is a ribonucleoprotein complex that co-translational targeting of proteins to appropriate membranes and secretory pathways, SRP dysregulation has been extensively reported in

several cancers [12]. The intimate connection between LRRC59 and SRP pathway implied that LRRC59 plays a direct role in the promotion of OSCC cell proliferation and metastasis through its action in the SRP pathway and/or translation of secretory/membrane proteins.

The type II transmembrane protein CKAP4 was initially found in the ER, where it associates with microtubules to coordinate the overall ER organization [21]. Recent research has shown that CKAP4, which is released with exosomes, is crucial for cancer metastasis. For example, Song et al. showed that CKAP4-containing exosomes promote and sustain the stem-like phenotype as well as metastasis in NSCLC [13]. According to studies by Hirokazu et al. exosomal CKAP4 may serve as a biomarker for PDAC, and anti-CKAP4 mAbs may be beneficial in the establishment of a molecularly targeted therapy [14]. In this study, we found that LRRC59 strongly interacted with CKAP4. Additionally, our data showed that CKAP4 was upregulated in OSCC tissues and that its expression was positively correlated with LRRC59. LRRC59 knockdown significantly decreased CKAP4 levels in exosome secreted from OSCC cells. Moreover, our results revealed that exosomes secreted from OSCC cells, not LRRC59 depletion OSCC cells, could significantly promote the malignant migration of OSCC cells. We therefore demonstrated that LRRC59 enhanced the generation of CKAP4-cantained exosomes and contributes to migration and invasion in OSCC cells.

5. Conclusion

In summary, our results indicate that LRRC59 engage in the SRP pathway and/or secretory/membrane proteins translation as a direct role in the promotion OSCC cell proliferation, migration, and invasion. Moreover, we provide evidence that LRRC59 enhances the secretion of exosomes containing CKAP4 contributing to migration and invasion in OSCC cells. Therefore, based on our findings, LRRC59 may serve as a potential biomarker for OSCC patients, and LRRC59-induced exosome secretion via the CKAP4 axis may serve as a potential therapeutic target for OSCC.

Ethics approval and consent to participate

This study was approved by the Shanghai outdo Ethical Committee (approval no. 1907008).

Consent for publication

All patients provided written informed consent prior to enrollment in the study.

Availability of data and materials

This published article and its additional information files contain all the data generated or analyzed during this study. Other data supporting this subject's findings can be requested from the relevant author in compliance with reasonable regulations.

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CRediT authorship contribution statement

Qijun Sun: Writing – original draft, Investigation, Formal analysis, Data curation. **Lili Jin:** Writing – review & editing, Investigation, Funding acquisition, Formal analysis, Data curation. **Shunli Dong:** Writing – review & editing, Formal analysis, Data curation. **Ling Zhang:** Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e28083.

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