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Porphyromonas gingivalis outer membrane vesicles augments proliferation and metastasis of oral squamous cell carcinoma cells

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Abstract

Background *Porphyromonas gingivalis* (*P. gingivalis*) is closely related to Oral squamous cell carcinoma (OSCC), and *P. gingivalis* outer membrane vesicles (OMVs) is the main pathogenic factor, which is associated with periodontitis, atherosclerosis and other diseases. However, few studies have reported an association between *P. gingivalis* OMVs and OSCC. The purpose of this study was to establish the clinical relationship between *P. gingivalis* and OSCC based on clinical samples. Further, the effect of *P. gingivalis* OMVs on OSCC was observed with cell model in vitro, and the possible molecular mechanism was discussed.

Methods Immunohistochemistry was used to detect the abundance of *P. gingivalis* in OSCC and its paired paracancer tissues, and to analyze the correlation between *P. gingivalis* and clinicopathological parameters of patients. *P. gingivalis* OMVs were isolated to observe its effects on the proliferation and migration of OSCC cell lines. RNA-seq was performed and the expression of differentially expressed genes (DEGs) was detected by real-time quantitative PCR (RT-qPCR) to explore the potential mechnism of *P. gingivalis* OMVs on OSCC progression.

Results The abundance of *P. gingivalis* in OSCC was higher than that in para-cancerous tissues, and was positively correlated with the degree of tissue differentiation ($P=0.028$), T stage ($P<0.001$), and clinical stage ($P=0.011$). *P. gingivalis* OMVs promoted the proliferation and migration of HN6 cells, and promoted the proliferation of CAL27 cells, but had no significant effect on its migration. *P. gingivalis* OMVs treatment attenuated the expressions of TNFSF15, ZNF292, ATRX, ASPM and KIF20B in CAL27 and HN6 cells.

Conclusion This study suggests that *P. gingivalis* may be an indicator of poor prognosis for OSCC. *P. gingivalis* OMVs may down-regulate the expression of TNFSF15, ZNF292, ATRX, ASPM, KIF20B and participate in the occurrence and development of OSCC.

Keywords Squamous cell carcinoma of head and neck, *Porphyromonas gingivalis*, Extracelular vesicles

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Background

Oral cancer is the most common malignancy in the head and neck region, with over 90% of cases being of the squamous cell carcinoma histopathological type [1, 2]. Oral squamous cell carcinoma (OSCC) is characterized by its insidious nature, often diagnosed at an advanced stage, accompanied by impairments in speech and swallowing. OSCC exhibits high invasive and metastatic potential, frequently leading to lymph node metastasis. The 5-year survival rate is approximately 50-60%, indicating a poor prognosis with a high recurrence rate [3–6].

OSCC is the result of multifactorial interactions, and well-established risk factors for OSCC include tobacco, alcohol, betel nut, HPV infection, etc [7]. In recent years, new perspectives suggest that periodontitis may also be a risk factor for OSCC [8, 9]. Still, some studies indicate that approximately 15% of the etiology of OSCC cannot be explained by the currently identified risk factors for OSCC [10]. Therefore, there is an urgent need to explore new potential pathogenic factors as targets for the prevention or treatment of OSCC. Epidemiological studies indicate that around 20% of cancers are associated with microbial infections [11, 12], such as Fusobacterium nucleatum with colorectal cancer [13], Salmonella with gallbladder cancer [14], etc. The oral microbiome is one of the most complex microbial communities in the human body, with over 700 bacterial species residing [15]. Increasing research suggests a close association between the oral microbiome and the occurrence and development of OSCC [16, 17]. There are differences in the oral microbial communities between OSCC patients and healthy individuals, with an increased proportion of periodontal pathogens, including Porphyromonas gingivalis (*P. gingivalis*), in the oral cavity of OSCC patients [18]. As a key pathogenic bacterium in periodontal diseases, numerous studies have demonstrated a close relationship between *P. gingivalis* and OSCC [18, 19], considering *P. gingivalis* as a potential risk factor in the occurrence and development of OSCC. Current research generally agrees that *P. gingivalis* mainly promotes oral cancer by stimulating cell proliferation, regulating apoptosis, enhancing cell migration and invasion, inducing chronic inflammation, immune evasion, and promoting angiogenesis, contributing to the initiation and progression of oral cancer [1, 20].

Outer membrane vesicles (OMVs) are spherical double-layered structures with sizes ranging from 50 to 250 nm, derived from Gram-negative bacteria through outer membrane shedding, and they play a crucial role in bacterial survival and exerting toxicity [21]. OMVs can be targeted for transport to host cells, mediating the entry of bacterial virulence factors and genetic material into host cells, regulating host cell functions, serving as bacterial renades delivered into the host [22, 23]. *P. gingivalis*, a member of the red complex associated with periodontitis, is a Gram-negative anaerobic bacterium that secretes OMVs at all stages of its growth. *P. gingivalis* concentrates virulence factors in the form of OMVs, and *P. gingivalis* OMVs exhibit significant functional advantages: stronger induction of inflammation, greater immunogenicity, and notable immunomodulatory effects compared to the parent bacteria [24–27]. OMVs produced by *P. gingivalis* entering host target organs can induce related diseases, such as periodontitis [28], Alzheimer's disease [29], cardiovascular diseases [30], and so on.

P. gingivalis is considered as an independent microbial risk factor associated with an increased mortality rate for cancers of the oral digestive system [31]. However, there is currently limited research discussing the correlation between *P. gingivalis* OMVs, serving as an effective carrier of *P. gingivalis* pathogenicity, and OSCC.

Therefore, this study collected 49 pairs of OSCC specimens for the detection of *P. gingivalis* abundance, aiming to establish the clinical association between *P. gingivalis* and OSCC. Subsequently, *P. gingivalis* OMVs were extracted and used to treat OSCC cells to investigate the impact of *P. gingivalis* OMVs on the biological behavior of OSCC cells. Transcriptome sequencing was employed to preliminarily explore the potential molecular mechanisms of *P. gingivalis* OMVs in the occurrence and development of OSCC, with the hope of providing new insights into the etiology and prevention of OSCC.

Methods

Collection of clinical samples

We collected tumor tissues and corresponding adjacent non-tumor tissues, as well as relevant clinical data, from 49 patients with oral squamous cell carcinoma treated at the First Affiliated Hospital of Fujian Medical University. Clinical pathological grading and staging were determined according to the 8th edition of the American Joint Committee for Cancer (AJCC) staging guidelines for oral cancer. Inclusion criteria for patients were as follows: absence of other malignant tumors, first-time hospital admission for oral tumors, no prior radiotherapy, chemotherapy, or biological therapy, absence of severe systemic infectious diseases, pathological confirmation of squamous cell carcinoma, and location of lesion tissues in the tongue, cheek, lip, floor of the mouth, gums, or other sites. Tumor tissue specimens were obtained from surgically excised tumor tissues, and corresponding adjacent non-tumor tissue specimens were taken from areas near the surgical safety margin. Pathological confirmation ensured the absence of residual cancer tissue in the adjacent non-tumor specimens. The study was approved by the Ethics Committee of First Affiliated Hospital of Fujian Medical University ([2015]084 – 1、[2015]084 – 2),

and conducted in accordance with the Declaration of Helsinki.

Detection of bacteria by immunohistochemistry

Immunohistochemistry (IHC) were employed to assess the abundance of *P. gingivalis* in cancer tissues and their corresponding adjacent non-tumor tissues. The analysis aimed to investigate the correlation between the detected levels of *P. gingivalis* in cancer tissues and the clinical-pathological parameters of the patients. Paraffin-embedded specimens from 49 pairs of OSCC cancer tissues and their corresponding adjacent non-tumor tissues were sectioned into 4 μm slices for immunohistochemical staining. Sections were incubated with a primary antibody against *P. gingivalis* (1:200; 2116, Dai-An, China) overnight at 4℃. Image acquisition of the sections was performed using the Easyscan slide scanner. The immunohistochemical staining for *P. gingivalis* was scored by multiplying the intensity score (0 = no staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining) by the percentage of positive stained cells (0 = less than 10% positive cells, 1 = 10–30% positive cells, 2 = 30–60% positive cells, and 3 ≥ 60% positive cells). A score of ≥ 2 was used as the criterion for positive *P. gingivalis* staining [32]. Using the median value of the immunohistochemical score for *P. gingivalis* as the cutoff, all OSCC patients were classified into either the weak staining group or the strong staining group for *P. gingivalis*.

Bacteria and cells culture

The human OSCC cell lines CAL27 and HN6 were grown and cultured in high-glucose Dulbecco's modified Eagle's media (DMEM; SH30022.01, HyClone, America) supplemented with 10% fetal bovine serum (FBS; ST30-3302, PAN SERATECH, Germany) at 37 °C in 5% CO_2. *P. gingivalis* ATCC 33,277 was inoculated on freshly prepared BHI(CM1135, OXOID, UK) agar plates containing 0.05% yeast extract 0.1% vitamin K1, 0.5% hemin and 5% aseptic defibrinated sheep blood, and passaged in 37℃ anaerobic culture for 3–5 days. The colony was scraped with a sterile ring and cultured anaerobic in BHI bacterial liquid medium containing 0.05% yeast extract, 0.1% vitamin K 1 and 0.5% hemin.

Isolation and identification of *P. gingivalis* OMVs

P. gingivalis OMVs were extracted from the bacterial solution of logarithmic growth stage according to the instructions of ExoBacteria™ OMV Isolation Kit for Gram Negative Bacteria (System Biosciences, America). The extracted *P. gingivalis* OMVs was stored at -80℃ until needed.

The morphology and size of *P. gingivalis* OMVs were observed and photographed using transmission electron microscopy (Hitachi, Japan). The particle size and concentration of *P. gingivalis* OMVs were measured by nanoparticle tracking analyzer (PARTICLE METRIX; ZetaVIEW, Germany). OMV quantification was done using Bicinchoninic Acid Assay (P0010S, Beyotime, China).

Experimental grouping

Blank group: CAL27 and HN6 cells without any treatment;

10 μg/mL group: CAL27 and HN6 cells with 10 μg/mL *P. gingivalis* OMVs;

50 μg/mL group: CAL27 and HN6 cells with 50 μg/mL *P. gingivalis* OMVs;
Control-10 μg/mL group: The equivalent number of OMV Elution Buffer CAL27 and HN6 cells filtered by 0.22 μm filter were added when *P. gingivalis* OMVs action concentration was 10 μg/mL;

Control-50 μg/mL group: The equivalent number of OMV Elution Buffer CAL27 and HN6 cells filtered by 0.22 μm filter were added when *P. gingivalis* OMVs action concentration was 50 μg/mL.

Cell counting kit-8 (CCK-8) analysis

CAL27 and HN6 were inoculated on 96-well plates, the cells were evenly dispersed by gently shaking the plates, and then cultured in a constant temperature incubator at 37℃ and 5% CO_2. After 24 h, appropriate amount of *P. gingivalis* OMVs was added according to the above groups, and immediately 10 μL CCK-8 solution was added into the compound well and incubated in the cell incubator for 1 h away from light. The absorbance OD value of cells in each group at 450 nm was detected at 0 h, 24 h, 48 h, 72 h, 96 h and 120 h, and the average value of each group was taken. The growth curve was drawn with the time of adding *P. gingivalis* OMVs as the horizontal axis and OD value as the vertical axis.

EdU assay

CAL27 and HN6 cells were seeded in a 48-well plate and incubated overnight in a CO_2 incubator at 37℃. After 24 h, an appropriate amount of *P. gingivalis* OMVs was added to the cells according to the aforementioned groups. EdU labeling was performed 72 h later. The proliferating cell ratio was assessed following the instructions of the EdU assay kit (C6016M, UElandy, China).

Scratch healing assay

CAL27 and HN6 cells were seeded in a 12-well plate and incubated overnight in a CO_2 incubator at 37℃. After 24 h, an appropriate amount of *P. gingivalis* OMVs was added to the cells according to the aforementioned groups and treated for 48 h. A scratch wound was created

on the cell monolayer using the tip of a 200 μL pipette. The cells were gently washed three times with PBS to remove residual cells from the scratched area. Observation of cell migration area was performed at 0 h and 6 h for HN6, and at 0 h and 24 h for CAL27, using an inverted microscope (Zeiss, Germany). Images were captured, and the scratch healing area was quantified using Image J software. The formula used for calculating wound healing rate (%) was as follows: Wound Healing Rate (%) = (Area at 0 h - Area at 6–24 h) / Area at 0 h × 100%.

Transwell migration assay

Following the aforementioned grouping, CAL27 and HN6 cells treated for 48 h were suspended in 200 μL serum-free culture medium and added to the upper chamber of Transwell inserts. Subsequently, 600 μL of fresh culture medium containing 10% fetal bovine serum was added to the lower chamber, and the inserts were incubated in a CO_2 incubator for 24 h (HN6) to 48 h (CAL27). After removing the Transwell inserts, they were washed three times with PBS. Carefully wiping away the upper layer of cells on the membrane with a wet cotton swab, the cells were fixed with 4% paraformaldehyde for 20 min and stained with 0.1% crystal violet for 10 min. Excess crystal violet stain was removed by rinsing with PBS. Cell migration was observed under a 200× microscope, and images were captured.

RNA-seq

CAL27 and HN6 cells were seeded in a six-well plate and incubated overnight in a cell culture incubator. After that, an appropriate amount of *P. gingivalis* OMVs was added for a 24-hour treatment. The cells were then divided into the Blank group, Control-50 μg/mL group, and 50 μg/mL group according to the aforementioned grouping information. Subsequently, the cells were washed twice with pre-chilled PBS, and pre-chilled Trizol (Invitrogen, USA) was added. The samples were then submitted to Berry Genomics for RNA-seq analysis. Raw transcriptome reads had been deposited in the National Center for Biotechnology Information Sequence Read Archive under accession numbers PRJNA1107938 (https://www.ncbi.nlm.nih.gov/sra/PRJNA1107938).

Table 1 The primer sequences

Gene name	Primer	sequence (5′ to 3′)
GAPDH	Forward	TTGGTATCGTGGAAGGACTCA
	Reverse	AGTAGAGGCAGGGATGATGTT
TNFSF15	Forward	ACTCCCAGGTCACATTCCGT
	Reverse	CTTCGCATACAGACTTGGTCC
NPPA	Forward	CAACGCAGACCTGATGGATT
	Reverse	GCTTCTTCATTCGGCTCACT
ZNF292	Forward	GCTGTGTCCATTAAGGTGTCTC
	Reverse	CAAGTATCTTCAACTGGCGTATGT
ASPM	Forward	ACACCTGTAAGGACCAGAATAGT
	Reverse	CCAAGCGTATCCATCACCATT
ATRX	Forward	GTGTGACAGCAGTGAGGATTC
	Reverse	TGGCTTGTCTACTTAATGCTAACG
KIF20B	Forward	CAGCAAGATGAACCACCAGC
	Reverse	TTCTGCAATGTTCGGTCGCA
ANKRD12	Forward	GAGATTCAGCCATACAGTTCAGAG
	Reverse	TGCTTGAGGAATCACACTACAC
BRCA2	Forward	CAGCAGACCCAGCTTACCTT
	Reverse	TACGCAACTTCCACACGGTT
GCC2	Forward	GCAATTAGACGCTACCACTGA
	Reverse	TTCAATCTGGCACATCAACTCTT

Selection of differentially expressed genes

Two methods were employed to exclude the influence of OMV Elution Buffer and identify common Differentially expressed genes (DEGs) in both cell lines.:(1) Differential gene expression was separately compared between the 50 μg/mL group and the Control-50 μg/mL group in CAL27 and HN6 cells. The overlapping DEGs in both cell lines were then compiled. (2) The differential genes of the Control-50 μg/mLgroup and the Blank group in CAL27 and HN6 cells were counted respectively, and the obtained DEGs were deducted from the DEGs of the 50 μg/mL group and the Blank group, and then the intersection of the two cell lines was counted.

Then, the obtained DEGs were combined and further filtered using the UALCAN database to identify candidate genes.

Quantitative reverse transcription-PCR analysis of mRNAs

Total RNA was extracted using TRIzol reagent (15596-018, Invitrogen, America) according to the manufacturer's protocol. The RNA quality and purity were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher, USA). Subsequently, cDNA synthesis was carried out using the PrimeScript™ RT reagent Kit (RR047A, Takara, Japan) as per the manufacturer's instructions. Real-time quantitative PCR was performed using TB Green® Premix Ex Taq™ (RR420A, Takara, Japan) according to the manufacturer's protocol. For the RT-PCR reaction, a mixture of 10 μl SYBR Premix Ex TaqTM (2×), 0.8 μL PCR primer mix, 2 μL diluted template cDNA, 0.4 μL ROX Reference Dye, and 6.4 ml DEPC H_2O was prepared. The expression levels were normalized to the reference gene GAPDH, and the $2^{-(\Delta\Delta Ct)}$ method was used to compare the expression levels of the target genes in each group. The primers used in the study are listed in Table 1.

Statistical analysis

SPSS 27.0.1 software(Statistical Product and Service Solutions, Chicago, USA) was used for statistical data analysis. The abundance difference of *P.*

gingivalis between OSCC cancer tissues and adjacent tissues was analyzed by wilcoxon test, and the abundance of *P. gingivalis* and clinicopathological parameters of OSCC patients were analyzed by Chi-square test, and the correlation analysis was conducted by spearman grade correlation analysis. All experimental data were expressed as Mean ± SD. One-way analysis of variance (ANOVA) with LSD multiple comparison tests were applied to calculate the significance. Results were considered statistically significant when $P < 0.05$.

Results

Increased abundance of *P. gingivalis* in OSCC cancer tissues

We performed IHC on 49 cases of OSCC cancer tissues and corresponding adjacent non-cancerous tissues (Fig. 1A-I). The positivity rates of *P. gingivalis* in OSCC cancer tissues and adjacent non-cancerous tissues were 85.714% and 8.163%, respectively. The abundance of *P. gingivalis* in OSCC cancer tissues was significantly higher than that in adjacent non-cancerous tissues ($P<0.01$) (Fig. 1J). Positive staining for *P. gingivalis* was localized in the cytoplasm, particularly concentrated in the cancer nests, with expression observed in all stages and adjacent non-cancerous tissues.

P. gingivalis infection is associated with poor prognosis in OSCC patients

We collected a total of 49 specimens, with an age range of 30–84 years and a median age of 59 years. Based on IHC scores, all OSCC patients were classified into either low abundance (20/49) or high abundance (29/49) groups of *P. gingivalis*. Further evaluation of the correlation between *P. gingivalis* abundance and clinical-pathological characteristics of OSCC patients revealed significant differences in tissue differentiation degree ($P = 0.028$), T stage ($P < 0.001$), and clinical stage ($P = 0.011$) in cancer tissues. No significant differences were observed in terms of age ($P = 0.721$), smoking ($P = 0.277$), alcohol consumption ($P = 0.801$), lymph node metastasis ($P = 0.721$), and tumor location on the gingiva ($P = 0.721$). Additionally, Spearman rank correlation analysis demonstrated a significant correlation between *P. gingivalis* content in cancer tissues and tissue differentiation degree, T stage, and clinical stage. The abundance of *P. gingivalis* was higher in poorly differentiated, T3-T4 stage and clinical stage III-IV squamous cell carcinoma tissues (Table 2). Our study suggests that *P. gingivalis* may serve as a risk factor for predicting the prognosis of OSCC.

Identification of *P. gingivalis* OMVs

TEM negative staining (Fig. 2A) observed the extracted material with a circular or semi-circular double-membraned vesicular structure, consistent with the characteristics of *P. gingivalis* OMVs under TEM.As indicated by the red arrows, the extracted vesicle morphologies in the TEM images exhibit spherical or elliptical shapes, and possess distinct membrane structures (blue arrows). NTA analysis (Fig. 2B) revealed that the average diameter of the extracted material was approximately 217.9 nm. These combined features confirm that the extracted

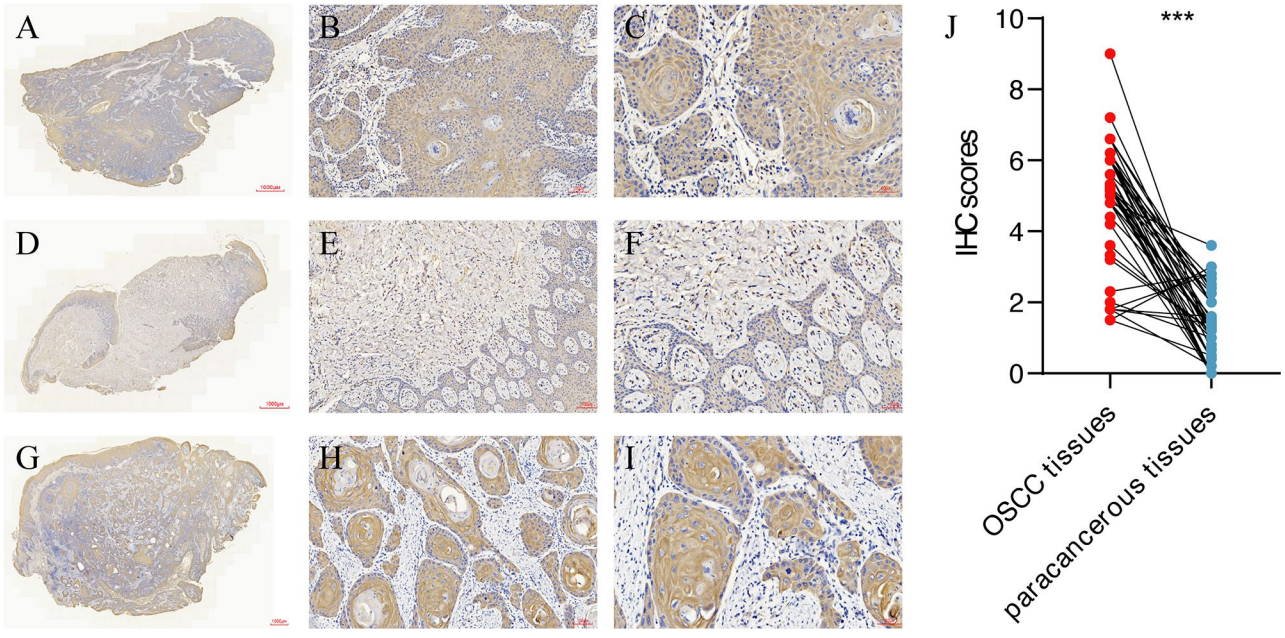

Fig. 1 The abundance of *P. gingivalis* in OSCC tissues was higher than that in paracancer tissues. (**A, D** and **G** are overall views of tissue sections, scale bar = 1000 μm; **B, E, H** scale bar = 100 μm; **C, F, I** scale bar = 60 μm). **A-C**: IHC image of *P. gingivalis* in OSCC cancer tissue; **D-F**: IHC images of *P. gingivalis* in paired paracancerous tissues; **G-I**: IHC image of *P. gingivalis* in cancerous nest; J: IHC scores of *P. gingivalis* in OSCC tissues and paracancerous tissues

Table 2 Correlation between *P. gingivalis* abundance and clinicopathological parameters in OSCC patients

	caseload	*P. gingivalis*		c^2	*P*-value	r_s	*P*-value
Variable		**Weak**	**Strong**				
caseload	49	20	29				
Age, years				0.127	0.721		
< 60	26	10	16				
≥ 60	23	10	13				
Tobacco consumption				1.18	0.277		
No	29	10	19				
Yes	20	10	10				
Alcohol consumption				0.063	0.801		
No	28	11	17				
Yes	21	9	12				
Tumor site				0.06	0.806		
Out site of gingiva	40	16	24				
Gingiva	9	4	5				
Differentiation				4.851	0.028*	0.315	0.028*
Well	18	11	7				
Moderate and poor	31	9	22				
T stage				13.013	< 0.001***	0.515	< 0.001***
T1-2	24	16	8				
T3-4	25	4	21				
N stage				0.203	0.652	0.064	0.660
N0	30	13	17				
N+	19	7	12				
Clinical stage				6.412	0.011*	0.362	0.011*
I-II	19	12	7				
III-IV	30	8	22				

* indicate $P < 0.05$,** indicate $P < 0.01$,*** indicate $P < 0.001$, signifying statistical significance

r_s: *spearman* correlation coefficient

c^2: Chi-square test

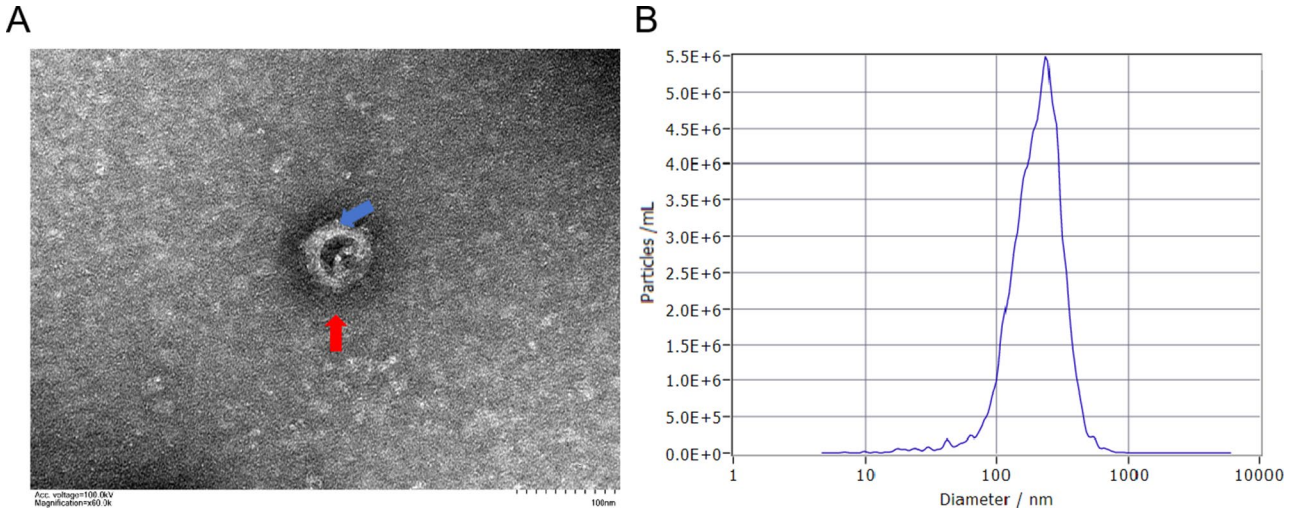

Fig. 2 Identification of *P. gingivalis* OMVs. **A**: TEM image of *P. gingivalis* OMVs (red arrows: indicate typical OMV structures, which are spherical or elliptical in shape; Blue arrows: the OMV membrane structures); **B**: NTA particle size distribution map

vesicles possess characteristics of *P. gingivalis* OMVs, thereby successfully isolating *P. gingivalis* OMVs.

P. gingivalis OMVs promotes the proliferation of oscc cells in vitro

In order to investigate the impact of *P. gingivalis* OMVs on OSCC cells, we treated CAL27 and HN6 cells with *P. gingivalis* OMVs (10 μg/mL group, 50 μg/mL group). The CCK-8 cell proliferation assay indicated that *P. gingivalis*

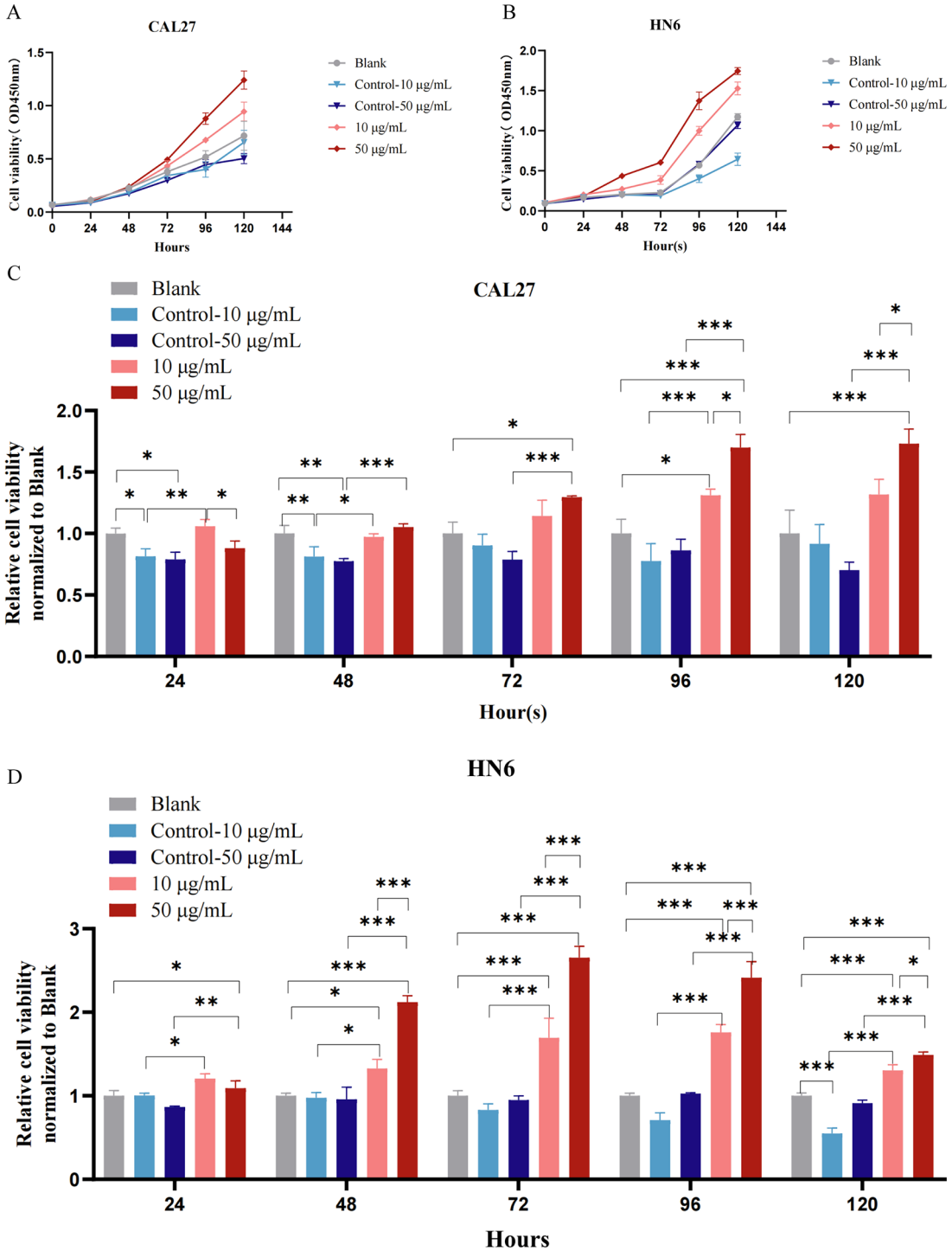

Fig. 3 *P. gingivalis* OMVs promotes the proliferation of CAL27 (**A, C**) and HN6 (**B, D**) cells in vitro (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$)

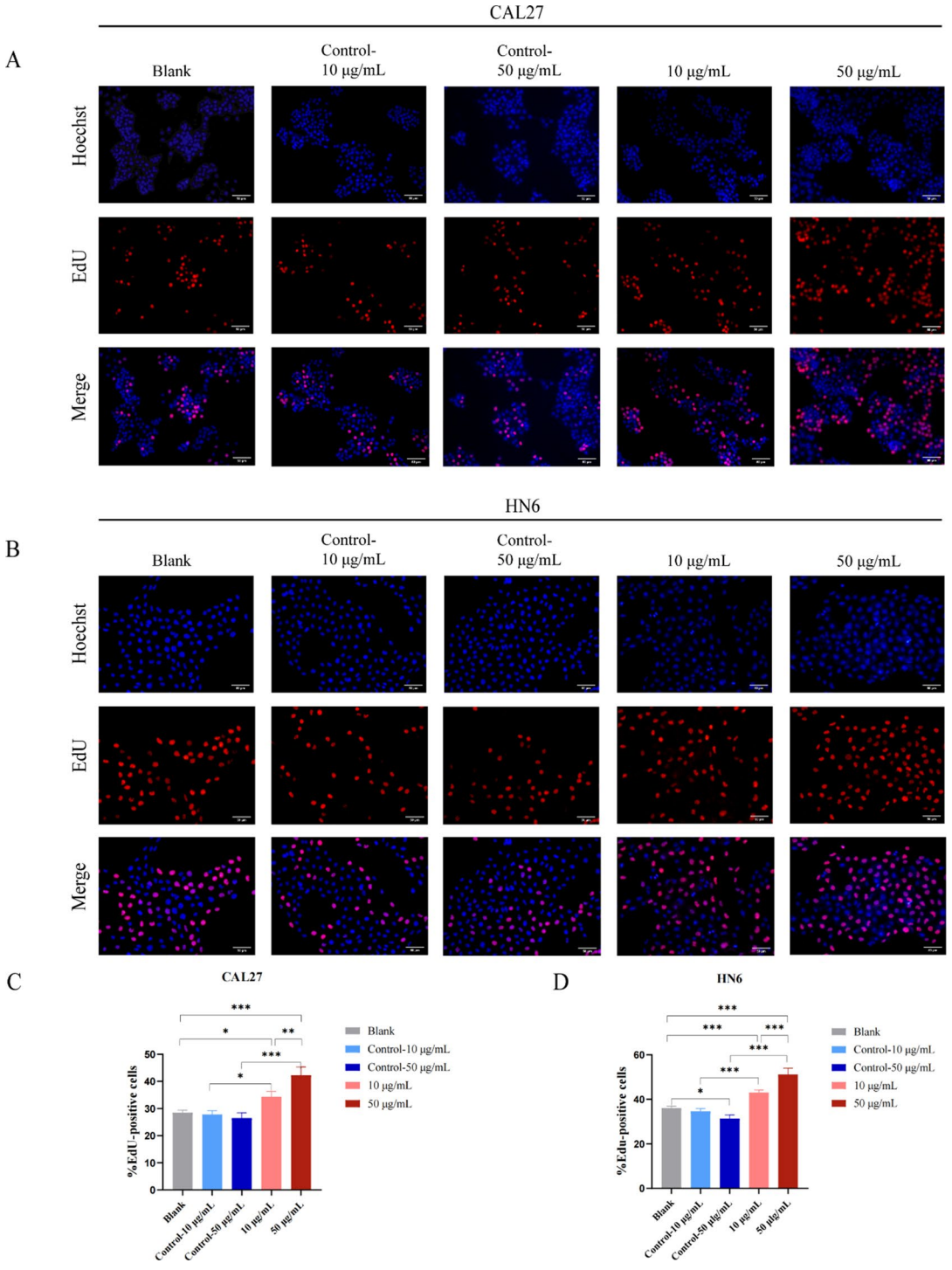

Fig. 4 EdU assay showed that *P. gingivalis* OMVs promoted the proliferation of CAL27 (**A, C**) and HN6 (**B, D**) cells. **A, C**: EdU expression in CAL27 cells at 72 h of treatment with *P. gingivalis* OMVs; **B, D**: EdU expression in HN6 cells at 72 h of treatment with *P. gingivalis* OMV. (200× magnification, scale bar = 50 μm, *:$P < 0.05$, **:$P < 0.01$, ***:$P < 0.001$)

OMVs promoted the proliferation of CAL27 and HN6 cells, with the most significant proliferative effect observed at 50 μg/mL of *P. gingivalis* OMVs (Fig. 3). The results of the EdU proliferation assay (Fig. 4) showed that the proliferation ratio of OSCC cells increased after treatment with *P. gingivalis* OMVs, and the 50 μg/mL group showed a higher proliferation rate than the 10 μg/mL group. The CCK-8 line chart (Fig. 3) showed that CAL27 and HN6 cells treated with *P. gingivalis* OMVs began to show significant improvement in proliferation ability after 48 h. Therefore, we selected CAL27 and HN6 cell models treated with *P. gingivalis* OMVs for 48 h in subsequent functional experiments.

P. gingivalis OMVs augments the migration of OSCC cells in vitro

After treating OSCC cells with *P. gingivalis* OMVs for 48 h, the effects of *P. gingivalis* OMVs on the horizontal migration ability of CAL27 and HN6 cells were investigated by cell scratch test (Fig. 5). The result showed that HN6 cells treated with *P. gingivalis* OMVs had a higher percentage of wound healing, Moreover, the wound healing percentage of HN6 cells treated with *P. gingivalis* OMVs at 50 μg/mL was higher than that in the 10 μg/mL group. In CAL27 cells, the results showed no significant difference in the percentage of wound healing among the groups.

We used Transwell chamber experiments (Fig. 6) to investigate the effect of *P. gingivalis* OMVs on the vertical migration ability of CAL27 and HN6 cells. The results showed that the number of HN6 cells treated with 10 μg/mL and 50 μg/mL *P. gingivalis* OMVs passing through the compartment increased significantly, and the number of HN6 cells treated with 50 μg/mL *P. gingivalis* OMVs passing through the compartment was the largest. In CAL27 cells, there was no significant difference in the number of cells passing through the compartment.

RNA-seq results

RNA-seq was performed to determine the possible molecular mechanism by which *P. gingivalis* OMVs promotes OSCC development. Differential gene expression analysis was conducted between the Blank group, the 50 μg/mL group, and the Control-50 μg/mL group. For the CAL27 cell line, there were 234 DEGs between the 50 μg/mL group and the Blank group, 193 DEGs between the 50 μg/mL group and the Control-50 μg/mL group, and 166 DEGs between the Control-50 μg/mL group and the Blank group. Regarding the HN6 cell line, there were 93 DEGs between the 50 μg/mL group and the Blank group, 38 DEGs between the 50 μg/mL group and the Control-50 μg/mL group, and 75 DEGs between the Control-50 μg/mL group and the Blank group (Fig. 7). The KEGG pathway enrichment analysis (Fig. 8) revealed that the DEGs in the CAL27 cell 50 μg/mL group versus

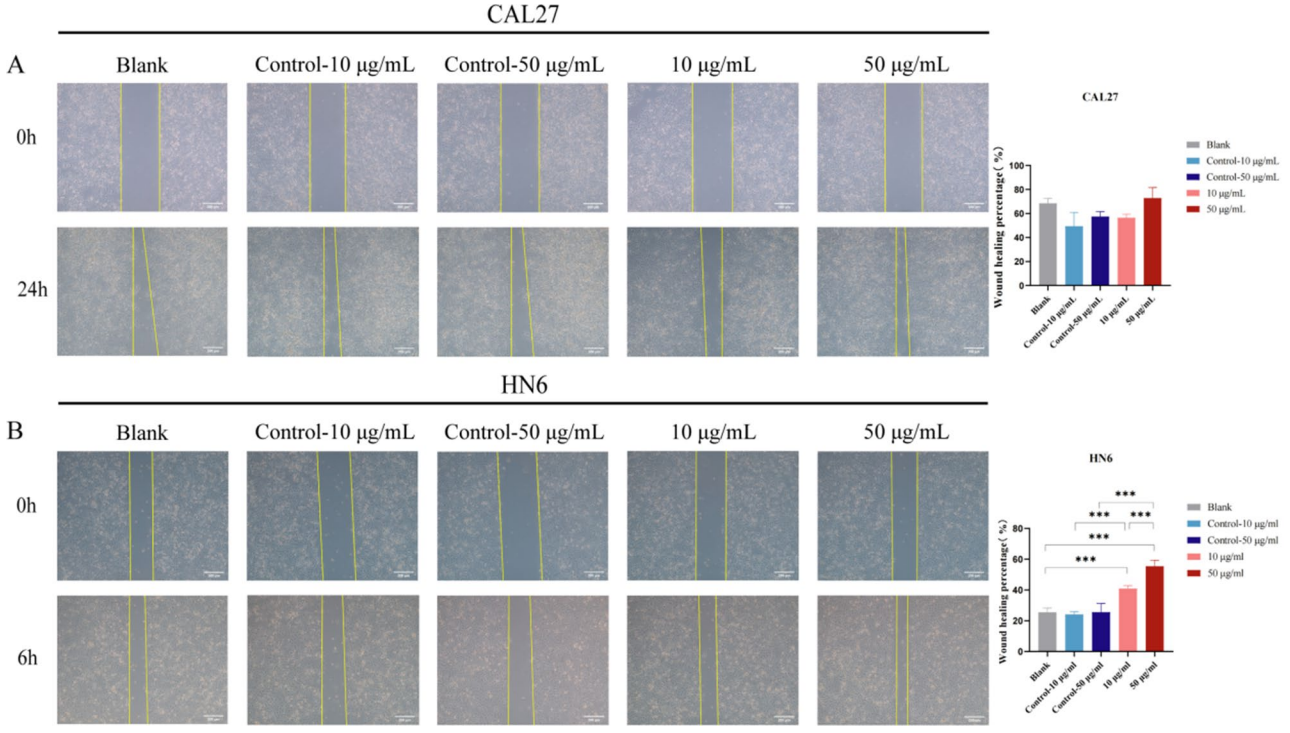

Fig. 5 *P. gingivalis* OMVs had no significant effect on the horizontal migration of CAL27 cells (**A**), but promoted the horizontal migration of HN6 cells (**B**), 50× magnification, scale bar = 200 μm, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$)

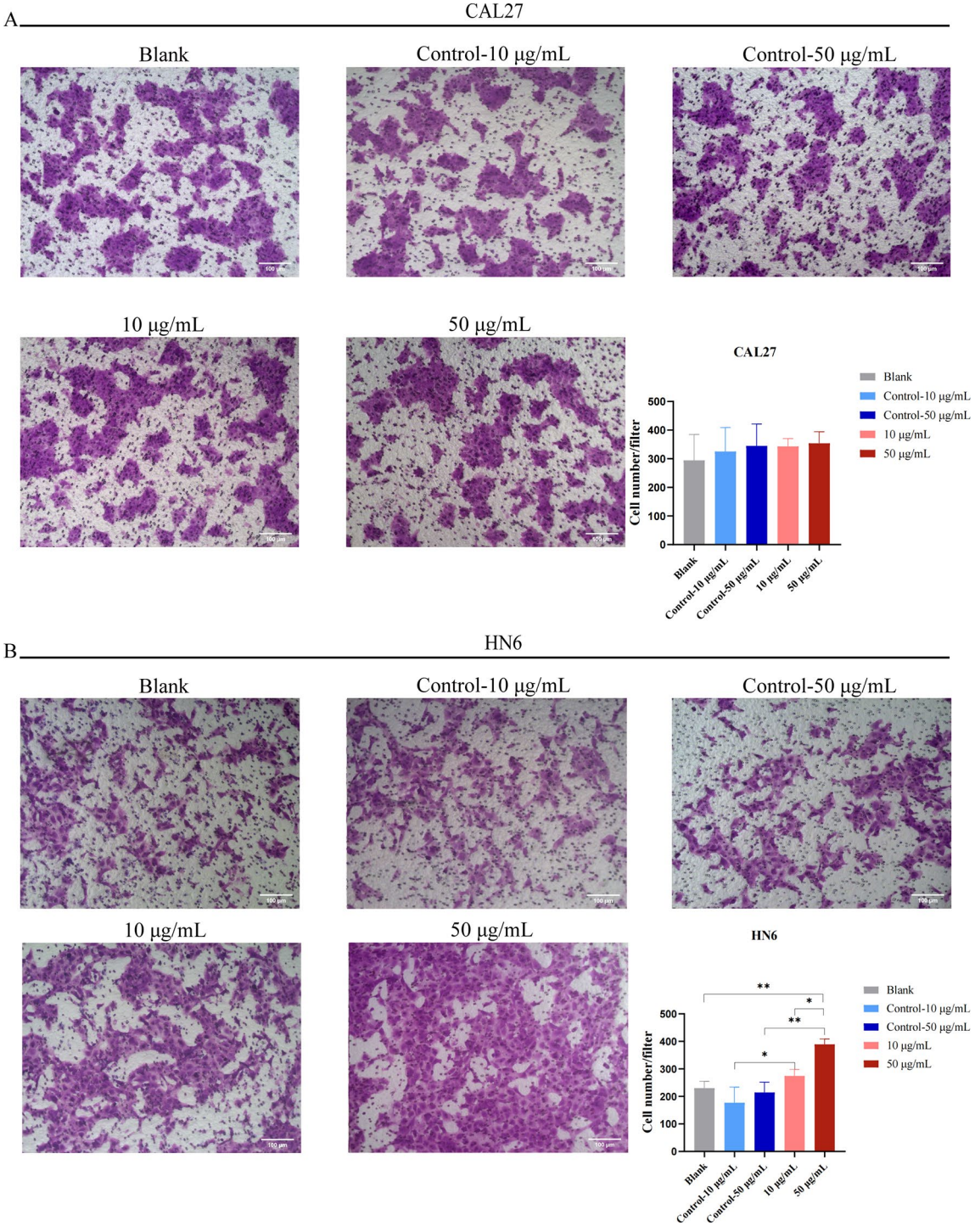

Fig. 6 *P. gingivalis* OMVs had no significant effect on the vertical migration of CAL27 cells (**A**), but promoted the vertical migration of HN6 cells (**B**) (100× magnification, scale bar = 200 μm, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$)

the Control-50 μg/mL group were mainly enriched in pathways such as cytokine-cytokine receptor interaction, transcriptional misregulation in cancer, and renin secretion. As for the HN6 cell 50 μg/mL group versus the Control-50 μg/mL group, the DEGs were predominantly enriched in pathways including the PI3K-Akt signaling pathway, cytokine-cytokine receptor interaction, and cell adhesion molecules.

Selection and validation of DEGs

The DEGs obtained by the above two intersection methods (Fig. 9A-B) are as follows: TNFSF15, NPPA, ZNF292,

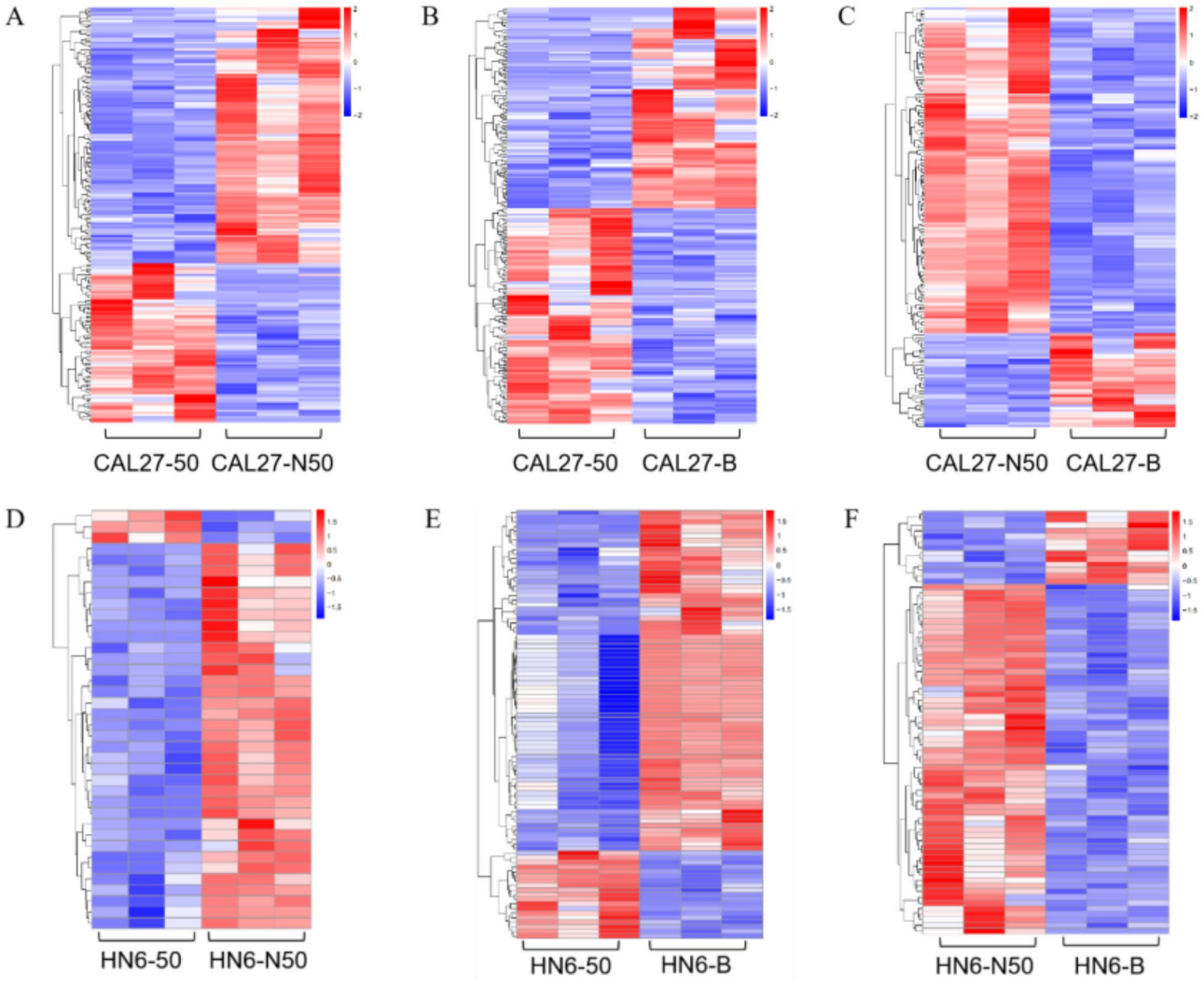

Fig. 7 DEGs heatmap. **A**: Cal27 cells 50 μg/mL versus Control-50 μg/mL; **B**: Cal27 cells 50 μg/mL versus Blank; **C**: Cal27 cells Control-50 μg/mL group versus Blank group; **D**: HN6 cells 50 μg/mL versus Control-50 μg/mL; **E**: HN6 cells 50 μg/mL versus Blank group, **F**. HN6 cells Control-50 μg/mL Group versus Blank group. Cal27(HN6)-B indicates the Blank group, Cal27(HN6)-N50 indicates the Control-50 μg/mL group, and CAL27(HN6) -50 indicates the 50 μg/mL Group. Red indicates high expression and blue indicates low expression

ASPM, ATRX, KIF20B, ANKRD12, BRCA2, GCC2, GOLGA4, FLRT3, CCDC88A, ANKRD26, NEB, TRIP11, EEA1, RSPH6A, SLCO1C1, GNG13. Subsequently, UALCAN database was used for survival analysis of these DEGs in HNSCC patients. The results showed (Fig. 9C-H) that HNSCC patients with low/medium expression of TNFSF15, ZNF292, KIF20B, ANKRD12, BRCA2 had lower survival rates, and the differences were statistically significant. HNSCC patients with low/medium expression of ASPM had lower survival rates in the first 5 years compared to those with high expression, but survival rates became similar thereafter. Considering the reported roles of these DEGs in other cancers, TNFSF15, NPPA, ZNF292, ASPM, ATRX, KIF20B, ANKRD12, BRCA2, GCC2 were selected for RT-qPCR validation. The RT-qPCR results (Fig. 10) showed that the expression of TNFSF15, ZNF292, ATRX, KIF20B, and ASPM genes in the 50 μg/mL group of CAL27 and HN6 cells was lower than in the Control-50 μg/mL group and Blank group. NPPA showed no significant difference in expression among the three groups in CAL27 and HN6 cells. ANKRD12, GCC2, and BRCA2 genes in the 50 μg/mL group of CAL27 cells were expressed lower than in the Blank group and Control-50 μg/mL group, while in HN6, no significant differences were observed in these three genes.

Discussion

Periodontitis and its pathogenic pathogen *P. gingivalis* are significantly positively correlated with the risk of oral cancer [33–36]. *P. gingivalis*, a gram-negative obligate anaerobic bacterium, is considered to be one of the

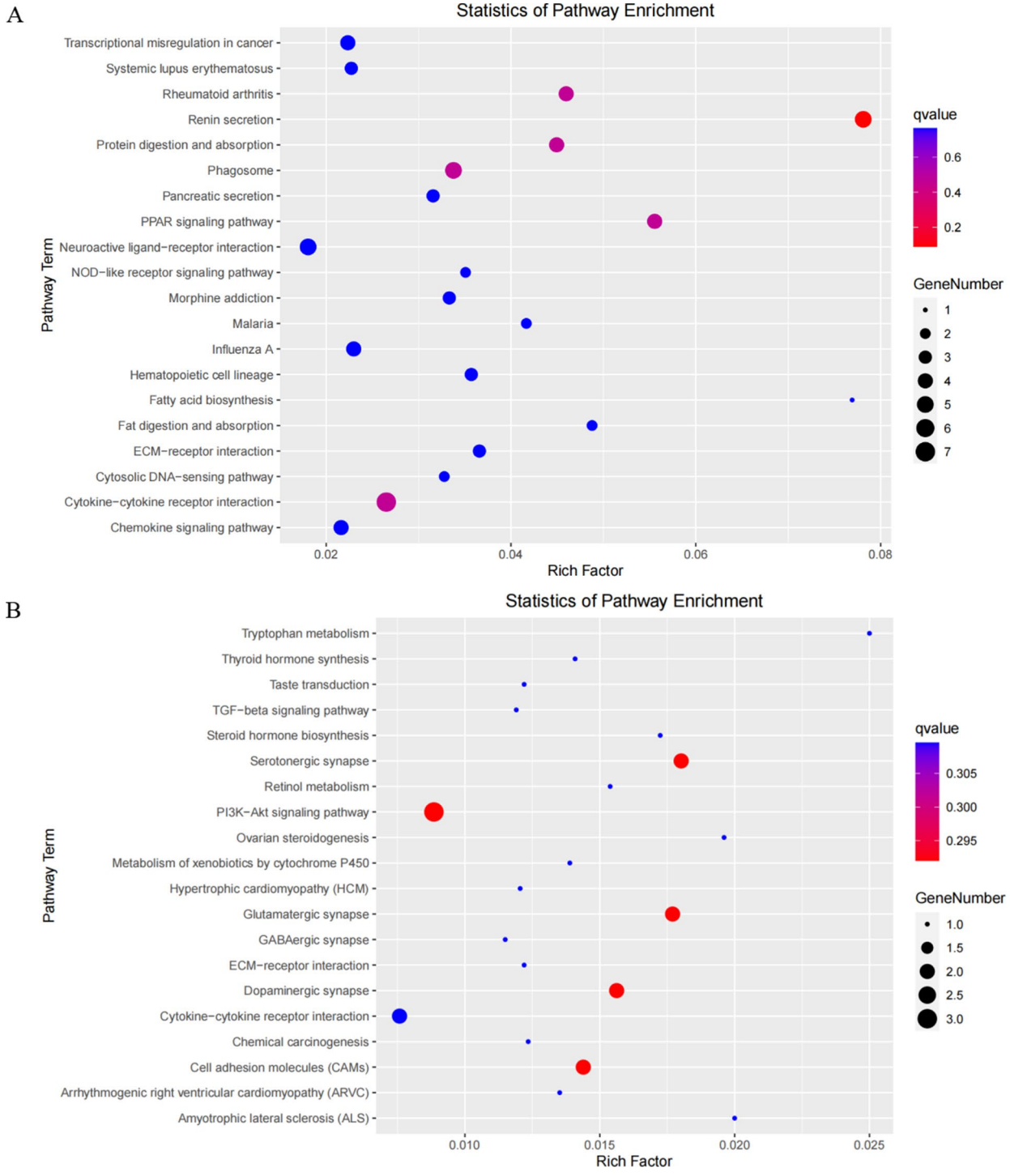

Fig. 8 KEGG pathway analysis of DEGs in the 50 μg/mL group versus the Control-50 μg/mL group. (**A**) CAL27; (**B**) HN6

key periodontal bacteria in the mouth with carcinogenic potential. *P. gingivalis* is closely related to oral, esophageal and pancreatic cancer, and is associated with poor prognosis of patients [18, 32, 37].

In this study, it was observed that the abundance of cytoplasmic *P. gingivalis* in OSCC cancer tissues was significantly higher than that in paracancer tissues. *P. gingivalis* existed in a wide range of OSCC cancer tissues, most of which were in the upper cortex. Positive staining of *P. gingivalis* can also be seen in some muscle layers, especially in cancer nests. These findings suggest that *P. gingivalis* may play an important role in the occurrence or progression of OSCC by invading into the tumor cells and adapting to the tumor microenvironment. In

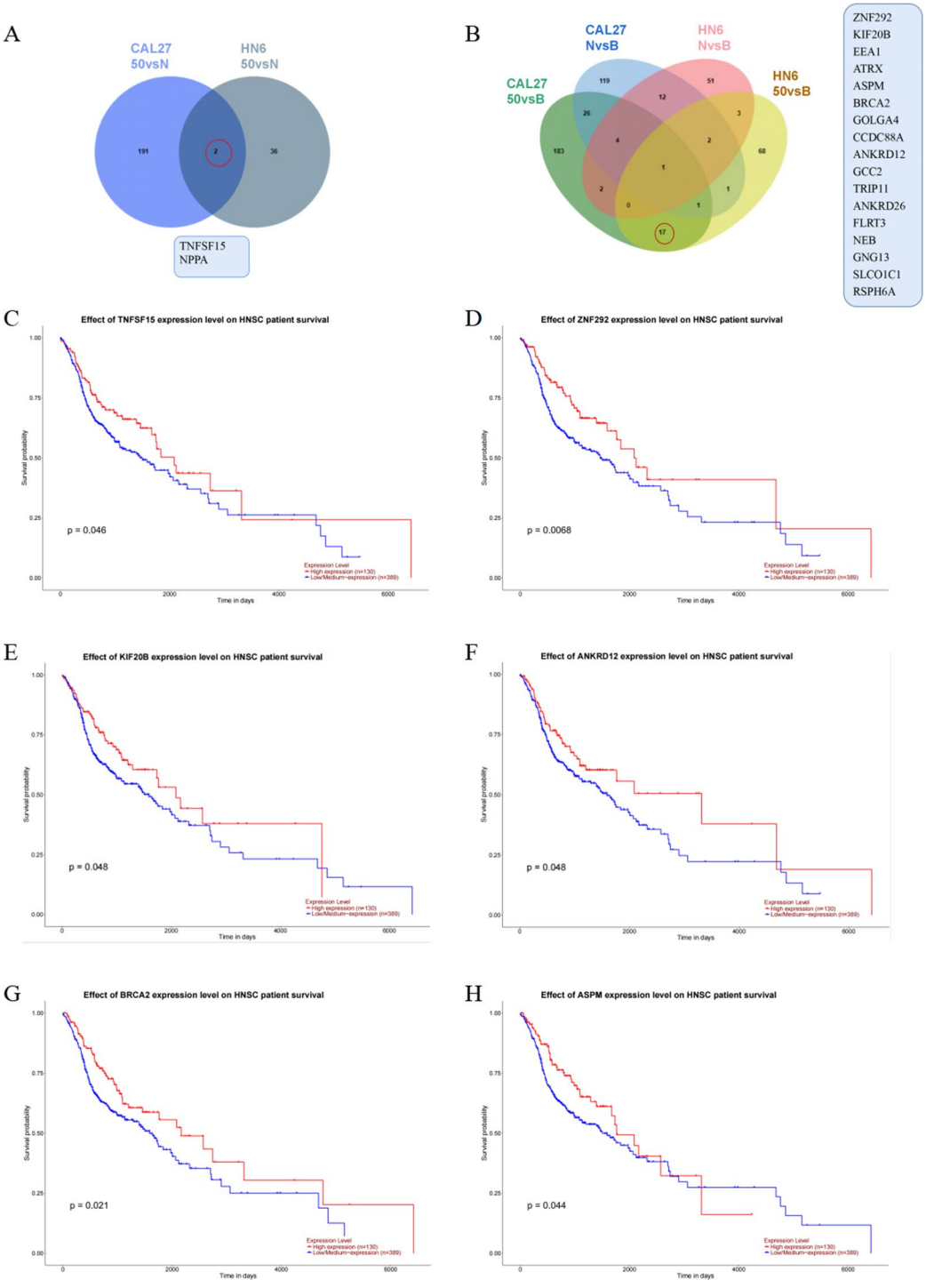

Fig. 9 Selection and validation of differentially expressed genes. **A**: The intersection of CAL27 cell DEGs (50 μg/mL group versus Control-50 μg/mL group) and HN6 cell DEGs (50 μg/mL group versus Control-50 μg/mL group); **B**: intersection of CAL27, HN6 cells in DEGs (50 μg/mL group versus Blank group) after subtracting genes that overlap with DEGs (Control-50 μg/mL group versus Blank group). Cal27(HN6)-**B** indicates the Blank group, Cal27(HN6)-N50 indicates the Control-50 μg/mL group, and CAL27(HN6) -50 indicates the 50 μg/mL Group; **C-H**: among the HNSCC patient information collected by TCGA, survival curve analysis by UALCAN revealed that low expression of TNFSF15 (**C**), ZNF292 (**D**), KIF20B (**E**), ANKRD12 (**F**), BRCA2 (**G**), and ASPM (**H**) was associated with survival of HNSCC patients. Red for high

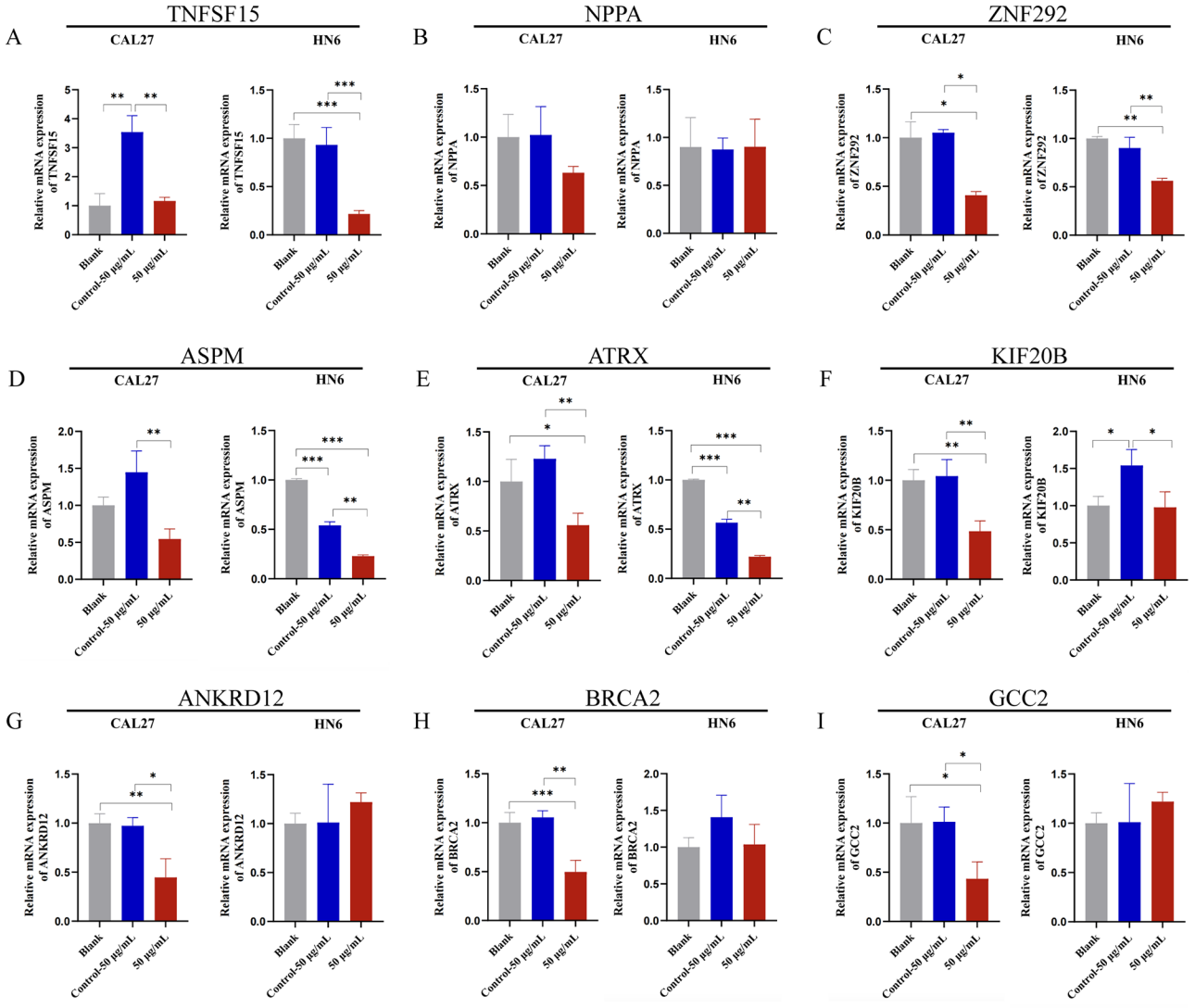

Fig. 10 *P. gingivalis* OMVs decreased the expression of TNFSF15, ZNF292, ATRX, KIF20B and ASPM genes in CAL27 and HN6 cells. **A**: TNFSF15; **B**: NPPA; **C**: ZNF292; **D**: ASPM; **E**: ATRX; **F**: KIF20B; **G**: ANKRD12; **H**: BRCA2; **I**: GCC2(*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$)

addition, this study found that the abundance of *P. gingivalis* was positively associated with poor prognosis of OSCC patients. Therefore, we speculate that improving oral hygiene, regulating the oral flora of patients, and changing the long-term colonization of *P. gingivalis* in the host may be an effective adjunct to conventional anticancer therapy.

All living organisms produce membrane-derived lipid bilayer Extracellular vesicles (EVs), also known as Membrane vesicles (MVs), with different cell targets [38]. *P. gingivalis* OMVs is derived from the extracorporeal membrane of bacteria and contains the virulence factor of *P. gingivalis*, which is composed of lipopolysaccharide, outer membrane protein, phospholipid, DNA and gingival protease [39, 40]. After bacterial OMVs are absorbed into host cells, they can affect the expression and regulation of host cell genes, change the function of host cells, and play an important role in various disease processes [41, 42]. The OMVs extracted in this study showed a circular or circular-like double-layer membrane vesicle structure under TEM negative influence. NTA analysis revealed that the average particle size of the extract was 217.9 nm, which was consistent with the theoretical particle size of *P. gingivalis* OMVs and consistent with previous literature reports [22]. The combined results of TEM and NTA indicate the successful extraction of *P. gingivalis* OMVs. In order to exclude the influence of OMV Elution Buffer during kit extraction, negative Control Control-10 μg/mL group and Control-50 μg/mL group were set up in this study. When the difference between the 10 μg/mL group and its corresponding negative Control Control-10 μg/mL group or the 50 μg/mL group and

its corresponding Control-50 μg/mL group is statistically significant, in other words, the changes of OSCC cells after *P. gingivalis* OMVs treatment in this experiment were not caused by the OMV Elution Buffer.

Most studies have shown that *P. gingivalis* can promote the proliferation of OSCC cells through various ways after infection [43, 44]. However, Cho et al. [45] found that *P. gingivalis* induced G1 cell cycle arrest after infection, inhibited the proliferation of OSCC cells, and enhanced their autophagy. Some scholars believe that *P. gingivalis* might cause both pro-apoptotic and anti-apoptotic phenotypes. *P. gingivalis* can mediate cell cycle arrest and autophagy to inhibit the proliferation of OSCC cells, and some products secreted by *P. gingivalis* promote the proliferation of OSCC cells [32]. However, there are few studies on the effect of *P. gingivalis* product OMVs on the proliferative phenotype of OSCC cells. In this study, we further revealed that *P. gingivalis* OMVs promoted the proliferation of CAL27 and HN6 cells in a dose-dependent manner. The uncontrolled proliferation of cancer cells is the most basic biological feature of malignant tumors. Therefore, it is speculated that *P. gingivalis* OMVs may play a role in the occurrence or development of OSCC by promoting the proliferation of cancer cells. The specific mechanism needs to be further studied.

Most studies have shown that *P. gingivalis* can enhance the migration and invasion of tumor cells mainly by promoting the occurrence of epithelial-mesenchymal transformation (EMT) and the expression of matrix metalloproteinases(MMPs). It has been reported that *P. gingivalis* OMVs can promote the migration and invasion of OSCC cell line HSC-3 cells in vitro [22]. In this study, it was observed that OMVs secreted by *P. gingivalis* enhanced the migration of HN6 cells in a dose-dependent manner, but had no significant effect on the migration of CAL27 cells. It is speculated that the possible reason is the specificity of cell type.

Studies have suggested that mutations in OSCC are mainly associated with tumor suppressor gene mutations [46]. ATRX plays a crucial role in maintaining genome stability and telomere regulation [47, 48]. Although low expression of ATRX in HNSCC patients does not show a significant correlation with poor prognosis in the UALCAN database, ATRX has been widely recognized as a tumor suppressor gene. Mutations or deletions in ATRX have been reported in various cancers, such as hepatocellular carcinoma [49] and glioma [50], highlighting its importance in tumor initiation and progression. TNFSF15 binds to endothelial cell surface-specific death receptor 3, inhibiting endothelial cell growth and suppressing angiogenesis. The downregulation of TNFSF15 expression in proliferating endothelial cells and tumor vessels has been reported [51, 52]. Studies have confirmed that TNFSF15 can inhibit tumor angiogenesis and, consequently, tumor growth [51]. Low expression of TNFSF15 is associated with poor prognosis in cancers such as breast cancer [53] and prostate cancer [52]. Overexpression of TNFSF15 promoted the polarization of macrophages towards M1 phenotype in mouse models of lung cancer, resulting in the reconstruction of tumor immune microenvironment and inhibiting tumor growth [54].Therefore, reduced expression of TNFSF15 may play a crucial role in cancer progression and metastasis. ZNF292 is a growth hormone-dependent zinc finger transcription factor, and some studies propose it as a tumor suppressor gene [55, 56]. Research has demonstrated that ZNF292 expression is lower in esophageal squamous cell carcinoma tissue than in adjacent and normal tissue, and decreased ZNF292 expression promotes the proliferation and cell cycle progression of esophageal squamous cell carcinoma cells [57]. Given the adjacent anatomical location and shared upper digestive tract classification, most studies indicate that *P. gingivalis* plays a pro-carcinogenic role in esophageal squamous cell carcinoma [58].Therefore, we hypothesized that the down-regulation of ZNF292 might be involved in the mechanism of action of *P. gingivalis* OMVs to promote the proliferation of CAL27 and HN6 cells. KIF20B has been reported to promote carcinogenesis in various cancers, including colorectal cancer [59] and hepatocellular carcinoma [60]. Studies have reported that reducing KIF20B expression inhibits the proliferation and migration of CAL27 cells [61], indicating a complex role in tumor biology. ASPM is a microtubule-associated protein that participates in cell division and mitosis, playing a crucial role in cell proliferation. ASPM is upregulated in many cancers, including ovarian cancer [62] and hepatocellular carcinoma [63], promotes cancer progression and is associated with poor patient prognosis. In summary, TNFSF15, ZNF292 and ATRX play the role of tumor suppressor genes in the process of various cancers, and reducing the expression of these genes can enhance the malignant biological behavior of cancer cells, while ASPM and KIF20B play the role of cancer promoting genes. Therefore, this study speculated that *P. gingivalis* OMVs might promote the malignant biological behavior of CAL27 and HN6 cells by decreasing the expression of TNFSF15, ZNF292 and ATRX. The biological behavior of Pgingivalis OMVs down-regulating TNFSF15, ZNF292 and ATRX gene expression may mask the effect of *P. gingivalis* OMVS down-regulating ASPM and KIF20B gene expression, but the specific mechanism is still unclear and needs further investigation. Downregulation of these genes may represent potential molecular mechanisms by which *P. gingivalis* OMVs promote the development of OSCC. Future research should focus on the functional mechanisms of these genes in OSCC and their interactions with P.

gingivalis OMVs, with the aim of identifying novel therapeutic targets and strategies for OSCC.

Just as the treatment of Helicobacter pylori and HPV has reduced the incidence of gastric and cervical cancer, it is expected that in the future, by regulating the oral flora, altering the long-term colonization of *P. gingivalis* in the host or inhibiting the secretion of P. gingivalis OMVs may be an effective adjunct to the prevention of oral cancer or conventional anticancer therapy.To achieve this, it is essential to further elucidate the specific mechanisms underlying the interaction between *P. gingivalis* and OSCC, as well as the critical role of OMVs in this process. Such research will aid in developing targeted interventions, such as specific antimicrobial therapies, vaccines, or immune modulatory strategies.

There are still some limitations in this study. First, the functional experiments in this study were all conducted on a two-dimensional plane(2D), and some growth characteristics of cells in a three-dimensional(3D) environment may be lost. Therefore, 3D culture or in vivo studies should be further explored in the future. Second, this study only validated the impact of *P. gingivalis* OMVs on differential gene expression, but the specific functions of these genes in *P. gingivalis* OMVs-mediated OSCC and their interaction networks require further investigation. Additionally, this study only explored the impact of P. gingivalis outer membrane vesicles OMV on OSCC at the overall level. Further analysis of more tissue samples is needed to clarify the specific effects of OMV on squamous cell carcinomas in different parts of the oral cavity. Finally, this study did not directly assess the therapeutic potential of *P. gingivalis* OMV inhibitors in vivo. Future research should establish suitable animal models to further reveal the in vivo effects of *P. gingivalis* OMVs on OSCC progression and explore whether OMV-targeted inhibitors can effectively suppress their pro-tumorigenic effects, potentially offering new therapeutic targets for OSCC. In addition, during the occurrence and development of periodontitis, it is often not the action of a single bacterium, but the result of the joint participation of multiple bacteria. As for the effects of the OMV of more periodontitis - related bacteria, such as *Aggregatibacter actinomycetemcomitans* (*Aa*), on OSCC and the underlying mechanisms, further research is still needed.

Conclusions

Based on clinical samples, this study established the clinical relationship between *P. gingivalis* and OSCC. Further, the effect of *P. gingivalis* OMVS on OSCC was observed using in vitro cell model as carrier, and the possible molecular mechanism was preliminarily discussed. The final conclusions are as follows:

1. The abundance of *P. gingivalis* in cancer tissues of OSCC patients is higher than that in paracancerous tissues, and is associated with poor prognosis in OSCC patients.
2. *P. gingivalis* OMVs can promote the proliferation and migration of CAL27 and HN6 cells to varying degrees.
3. The effect of *P. gingivalis* OMVs on OSCC cells may be caused by down-regulating the expression of TNFSF15, ZNF292, ATRX, KIF20B and ASPM genes.

Abbreviations

OSCC	Oral squamous cell carcinoma
HNSCC	Head and neck squamous cell carcinoma
P. gingivalis	*Porphyromonas gingivalis*
OMVs	Outer membrane vesicles
IHC	Immunohistochemistry
RT-qPCR	Real-time fluorescent quantitative polymerase chain reaction
CCK-8	Cell counting kit-8
DEGs	Differentially expressed genes
OD	Optical density
EdU	5-ethynyl-2′-deoxyuridine
RNA-seq	RNA sequencing
SD	Standard deviation
LSD	Least significant difference
TEM	Transmission electron microscope
NTA	Nanoparticle tracking analysis
KEGG	Kyoto encyclopedia of genes and genomes
Aa	*Aggregatibacter actinomycetemcomitans*

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Author contributions

Y Zeng, YY and XZ conceived the experiments. Y Zeng, YY, XS, Y Zhao, YT and SL performed the experiments. Y Zeng and YY collected and analyzed the data. Y Zeng wrote the initial draft. Y Zeng and YY modified the manuscript. All authors have read and approved the final manuscript.

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Data availability

The datasets generated and analyzed during the current study are available in the NCBI repository https://www.ncbi.nlm.nih.gov/sra/PRJNA1107938, accession number PRJNA1107938.

Declarations

Ethics approval and consent to participate

The study was approved by the Ethics Committee of First Affiliated Hospital of Fujian Medical University ([2015]084 – 1、[2015]084 – 2). We hereby declare that our research has strictly adhered to the ethical principles of the Helsinki Declaration, and all participants involved in the study have signed informed consent forms to ensure that their rights are fully protected.

Consent for publication

Not applicable.

Competing interests
The authors declare no competing interests.

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