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ORIGINAL ARTICLE

Tumor-derived *Prevotella intermedia* aggravates gastric cancer by enhancing Perilipin 3 expression

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Abstract

The indigenous microbial milieu within tumorous tissues exerts a pivotal influence on the genesis and advancement of gastric cancer (GC). This investigation scrutinizes the functions and molecular mechanisms attributed to *Prevotella intermedia* in the malignant evolution of GC. Isolation of *P. intermedia* from paired GC tissues was undertaken. Quantification of *P. intermedia* abundance in 102 tissues was accomplished using quantitative real-time PCR (qRT-PCR). Assessment of the biological effects of *P. intermedia* on GC cells was observed using culture medium supernatant. Furthermore, the protein profile of GC cells treated with tumor-derived *P. intermedia* was examined through label-free protein analysis. The functionality of perilipin 3 (PLIN3) was subsequently confirmed using shRNA. Our investigation revealed that the relative abundance of *P. intermedia* in tumor tissues significantly surpassed that of corresponding healthy tissues. The abundance of *P. intermedia* exhibited correlations with tumor differentiation ($p = 0.006$), perineural invasion ($p = 0.004$), omentum majus invasion ($p = 0.040$), and the survival duration of GC patients ($p = 0.042$). The supernatant derived from tumor-associated *P. intermedia* bolstered the proliferation, clone formation, migration, and invasion of GC cells. After indirect co-cultivation with tumor-derived *P. intermedia*, dysregulation of 34 proteins, including PLIN3, was discerned in GC cells. Knockdown of PLIN3 mitigated the malignancy instigated by *P. intermedia* in GC cells. Our findings posit that *P. intermedia* from the tumor microenvironment plays a substantial role in the malignant progression of GC via the modulation of PLIN3 expression. Moreover, the relative abundance of *P. intermedia* might serve as a potential biomarker for the diagnosis and prognosis of GC.

KEYWORDS
diagnosis, gastric cancer, PLIN3, *Prevotella intermedia*, prognosis

Abbreviations: EMT, epithelial–mesenchymal transition; GC, gastric cancer; ICLAS, International Council for Laboratory Animal Science; LC–MS/MS, liquid chromatography-tandem mass spectrometry; PLIN3, Perilipin 3; TCGA, Cancer Genome Atlas Program; TCMA, The Cancer Microbiome Atlas.

Wei Liang, Zhengyang Zhou, and Qizhao Gao contributed equally to this work.

1 | INTRODUCTION

Gastric cancer (GC) is the fifth most prevalent malignancy and the third leading cause of cancer-related mortality globally.[1] Cancer development is an intricate process influenced by various factors, including genetic susceptibility, endocrine disruptions, physical or chemical stimuli, and infection by specific microorganisms.[2,3] The local microbial environment within tumors plays a non-negligible role in regulating host epigenetics, intrastromal signal transduction, and cell transformation.[4]

Among microorganisms, *Helicobacter pylori* (*H. pylori*) infection emerges as an independent risk factor for GC, activating the host's cellular and humoral immunity through the CagA protein, resulting in DNA damage.[5] The use of antibiotics to eliminate *H. pylori* has been found to reduce GC risk by 75%.[6] Recent studies have also uncovered other species, such as *Streptococcus*, *Prevotella*, *Veronella*, *Clostridium*, *Haemophilus*, and *Neisseria*, associated with GC formation or progression.[7]

As a notable periodontal pathogen, *P. intermedia* has been linked to the development of oropharyngeal carcinoma, particularly oral squamous cell carcinoma.[8–10] Furthermore, a systematic review consistently associated *P. intermedia* and *Treponema denticola* with colorectal neoplasia.[11] Despite studies revealing alterations in the microbiota of GC tissues compared to matched normal tissues, including changes in the relative abundance of *Prevotella*, the specific role of *P. intermedia* in GC formation or progression and its related mechanisms remain largely unknown.[12] Further research is imperative to elucidate the potential effects of *P. intermedia* on GC and its underlying mechanisms.

Perilipin 3 (PLIN3) is a member of the perilipin droplet protein family reported to be involved in the formation and accumulation of lipid droplets. Disordered expression of PLIN3 has been linked to diseases such as obesity, diabetes, and insulin resistance.[13] Abnormal expression of PLIN3 has also been reported to affect the progression of malignant tumors, including prostate cancer,[14] pancreatic cancer,[15] serous ovarian cancer,[16] lung cancer,[17] and breast cancer.[18] However, the expression pattern of PLIN3 and its function in GC cells remain unknown.

The primary objective of this study was to elucidate the effects and the underlying molecular mechanism of tumor tissue-derived *P. intermedia* on the growth and metastasis of GC cells. We discovered that the culture supernatant of tumor-derived *P. intermedia* significantly promoted the proliferation, migration, and invasion of GC cells. This effect appears to be associated with the upregulation of PLIN3 expression in GC cells, as detected through label-free protein analysis.

2 | MATERIALS AND METHODS

2.1 | Patients and tissue samples

A total of 102 pathologically confirmed tumor tissues and paired healthy tissues, located at least 5 cm away from the tumor periphery, were collected from gastric cancer (GC) patients who underwent either total or partial gastrectomy at Suzhou Municipal Hospital between April 2013 and March 2016. All participants had not received any prior radiotherapy, chemotherapy, or additional adjuvant treatments before undergoing surgery. Informed consent was obtained from all patients or their respective family members.

2.2 | Bacterial strains and growth conditions

Prevotella intermedia strains were isolated from GC tumor tissues and paired with healthy tissues using plate marking separation methods on blood plates. Briefly, surgical specimens, comprising tumor and corresponding healthy tissues, were promptly placed in an enclosed environment equipped with an anaerobic generation system (Mitsubishi, Japan). Subsequently, tissues were inoculated onto blood plates within an anaerobic setting and cultured at 37°C in the anaerobic generation system.

2.3 | Bacterial DNA isolation

A single, isolated, and purified clone of *P. intermedia* was cultured in a medium containing hemin and vitamin K within an anaerobic generation system at 37°C. Bacterial genomic DNA was extracted using the DNA extraction kit from Omega Bio-tek (Norcross, GA, USA) following the manufacturer's instructions.

2.4 | Polymerase chain reaction

Amplification was conducted using 2XTaq Master Mix (Vazyme, Nanjing, China). The resulting products underwent agarose gel electrophoresis, and outcomes were observed and captured using a gel imaging system.

2.5 | Cell lines and cell culture

Human GC cell lines AGS, HGC-27, and MKN-45 (Guangzhou Cellcook Biotech, Guangzhou, China) were cultured in a complete medium consisting of 90% RPMI 1640 (Invitrogen, Carlsbad, CA) and 10% FBS in a 5% CO_2 atmosphere at 37°C.

2.6 | RNA extraction and quantitative real-time PCR

Total RNA extraction was performed using TRIzol Reagent (Invitrogen, Carlsbad, CA), and the HiFiScript cDNA Synthesis Kit (Cwbio, Beijing, China) was used for RNA-to-cDNA transcription. Subsequently, quantitative real-time PCR (qRT-PCR) was conducted using the RealSYBR Mixture (Cwbio) with a real-time PCR Detection System (LightCycler 480, Roche, Switzerland). The 16S sequence served as an internal reference.

2.7 | Cell growth curves and cell colony formation assay

Cells from each group were seeded into a 24-well plate at a density of 1×10^4 cells per well, with six wells per group. Subsequently, the cells were digested and counted every 24 h to generate growth curves using GraphPad 5.0. Additionally, cells from each group were seeded into a six-well plate (1×10^4 cells per well) and cultured in complete medium in a 5% CO_2 atmosphere at 37°C for 7 days. The culture medium was refreshed every 3 days during this period. Before image capture, the cells were fixed with 4% paraformaldehyde for 30 min at room temperature and stained with crystal violet for 15 min.

2.8 | Transwell migration and invasion assays

Transwell migration and invasion assays were conducted following our previously established protocol.[19] Cells from each group were seeded into the top chamber of Transwell chambers (Chemicon, Temecula, CA, USA) with 200 μL RPMI 1640 medium at a density of 2×10^4 cells per well for the migration assay and 1×10^5 cells per well for the invasion assay.

2.9 | Cell transfection

Cell transfection was performed according to our established protocol.[20] ShRNA vectors (Hanbio, Shanghai, China) were used to knock down PLIN3. The sequences were 5′-GATCCGAACAGAGCTAC TTCGTACCTCGAGGTACGAAGTAGCTCTGTTCTTTTTTD -3′ (sh-PLIN3-1) and 5′-GATCCGTCCTAAGCCTGATGGAAACTCGAGTT TCCATCAGGCTTAGGACTTTTTTG -3′ (sh-PLIN3-2).

2.10 | Western blot analysis

Western blot was conducted following our established protocol.[20] RIPA buffer (Beyotime, Shanghai, China) was used to lyse cells, and SDS-PAGE was performed to separate the proteins. The target proteins were then promptly electrophoretically transferred. Specific primary antibodies and secondary antibodies were applied to detect the target proteins. Finally, protein bands were developed using the Enhanced Chemiluminescence Kit (Thermo Fisher, Waltham, USA). GAPDH was used as an internal reference in this study.

2.11 | Label-free protein analysis

First, proteins were extracted from cells via RIPA Lysis and Extraction Buffer, and then protein concentration was measured using the BCA method. Subsequently, proteins underwent reductive alkylation and trypsin enzymatic hydrolysis. Liquid chromatography-tandem mass spectrometry (LC–MS/MS) analysis was conducted to obtain the raw file of the original mass spectrometry results. The data were analyzed using MaxQuant software (version 1.6.2.10) to obtain the identification results.

2.12 | Xenograft mouse model

A suspension of 2×10^6 MKN-45 was suspended in 100 μL PBS and subcutaneously injected into 5-week-old BALB/c nude mice after corresponding co-culture or knockdown. The nude mice were killed and the tumors were collected. This research follows the ethical guidelines of the International Council for Laboratory Animal Science (ICLAS) in animal use and care.

2.13 | Immunohistochemistry

Tumor segments were fixed with 4% paraformaldehyde overnight at 4°C and subsequently embedded in paraffin. The segments were then sectioned to a thickness of 4 μm. Dewaxing procedures were followed by blocking non-specific sites with a 5% BSA solution for 1 h. Subsequently, the sections were incubated overnight at 4°C with primary antibodies against Ki-67 (Wanleibio, Shenyang, China) or PLIN3 (Proteintech, Wuhan, China) and washed with PBS. EnVision+/HRP/Rb (DAKO, Glostrup, Denmark) was applied for 1 h at 37°C. The segments were then covered with 3,3′-Diaminobenzidine (3,3′-DAB, Maxim, Fuzhou, China) for 5 min and counterstained with hematoxylin for 30 s. Finally, sections were photographed with a TE2000-U camera (Nikon, Tokyo, Japan).

2.14 | Statistical analysis

In this study, statistical analyses were conducted using SPSS 22.0 (Chicago, IL, USA). All experiments were performed in triplicate, and values were recorded as mean ± SD. Paired groups were analyzed using Student's *t*-test, while ANOVA was used to assess statistical differences among the three groups of treated samples. The Pearson χ^2-test was used to explore the correlation between the relative abundance of *P. intermedia* and clinical pathological features. A *p*-value of <0.05 was considered statistically significant.

3 | RESULTS

3.1 | Detection of *Prevotella intermedia* in gastric cancer tumor tissues

To understand the role of microorganisms in GC tumor tissues, we used The Cancer Microbiome Atlas (TCMA, https://tcma.pratt.duke.edu/), a validated decontamination statistical model, to analyze the prevalence of species in 166 GC tissues.[21] The results revealed that, at the genus level, *Prevotella* exhibited the highest infection rate with

a relatively higher abundance (Figure 1A). Subsequently, microbiological culture of GC tumor tissues was conducted. Round, translucent, smooth-surfaced black colonies on the preliminary separated blood agar plate were consistent with the characteristics of the *Prevotella* genus. Mass spectrometry analysis identified the strain as *P. intermediate*. Moreover, *P. intermedia* was isolated from both GC tissues (*P. intermedia*-T) and paired healthy tissues (*P. intermedia*-N), forming similar colonies on the blood agar plates (Figure 1B). PCR using specific primers targeting *P. intermedia* resulted in the amplification of a fragment of approximately 350 bp (Figure 1C). Sequencing analysis confirmed that the amplified fragment matched a partial gene sequence of *P. intermedia* in the National Center for Biotechnology Information (NCBI; Figure 1D).

For further analysis, genomic DNA of the two bacterial strains was extracted and subjected to nucleic acid sequencing using second-generation sequencing technology. The results revealed that both strains matched the gene sequence of *P. intermedia*, with slight differences observed in some genes, such as *VgrG* (Sequences S1 and S2).

3.2 | *Prevotella intermedia* as a potential biomarker for gastric cancer diagnosis and prognosis

The relative abundance of *P. intermedia* in 102 pairs of GC tissues and paired healthy tissues was determined using qPCR. *P. intermedia* was upregulated in 63.73% (65/102) of tumor tissues compared to paired healthy tissues. Additionally, 55.88% (57/102) of GC tissues exhibited at least a twofold upregulation of *P. intermediate*. The results indicated that the relative abundance of *P. intermedia* in tumor tissues was significantly higher than that of matched healthy tissues (Figure 2A) and was associated with tumor differentiation ($p = 0.006$), perineural invasion ($p = 0.004$), and omentum majus invasion ($p = 0.040$) in GC patients (Table 1). Survival analysis revealed

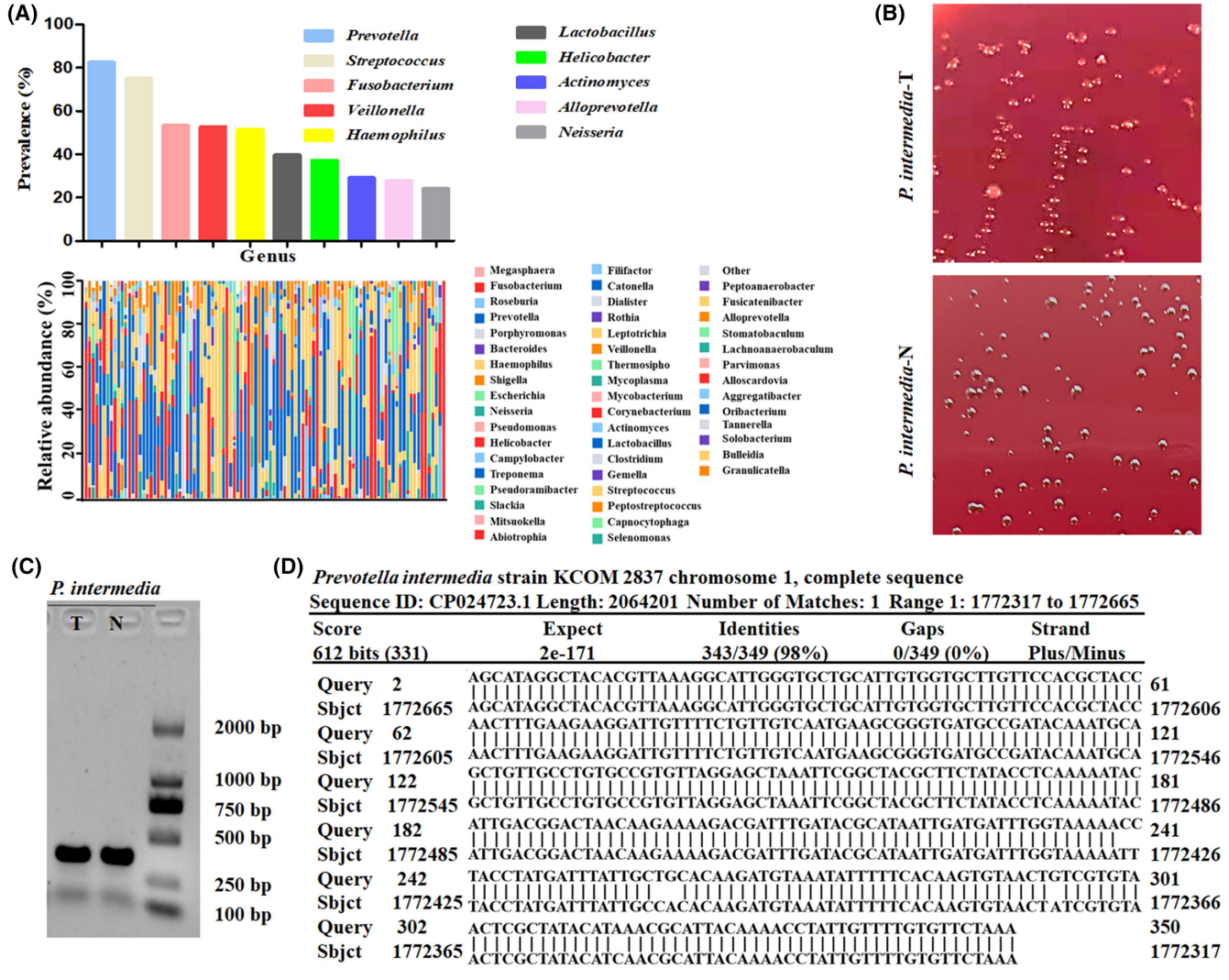


FIGURE 1 Isolation and identification of *Prevotella intermedia*. (A) Analysis of the prevalence of species in gastric cancer (GC) tissues. (B) Colony characteristic of *P. intermedia* on blood agar plate. *P. intermedia*-T, tumor tissue-derived *P. intermedia*; *P. intermedia*-N, normal tissue-derived *P. intermedia*. (C) Gel electrophoresis of PCR amplification products of *P. intermedia* colonies. (D) Sequence alignment of PCR amplification products of *P. intermedia* colonies.

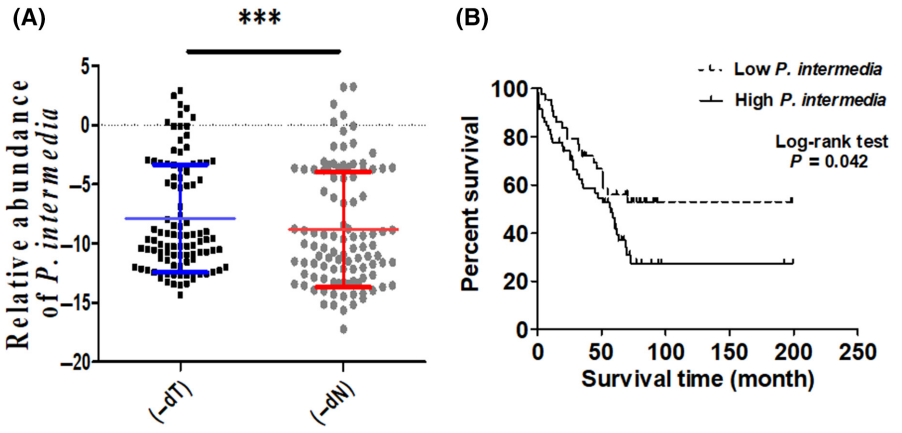


FIGURE 2 *Prevotella intermedia* was a potential biomarker for gastric cancer (GC) diagnosis and prognostic evaluation. (A) The relative abundance of *P. intermedia* in tumor tissues and matched healthy tissues. (B) Survival analysis of *P. intermedia* abundance in GC patients.

TABLE 1 The association between *Prevotella intermedia* expression levels (−ΔCt) in tumor tissues and the clinicopathological features of gastric cancer patients.

Features	Number	*Prevotella intermedia*		Mean ± SD	*p*-value	χ^2
		Low	High			
Gender						
Male	75	33	42	0.92 ± 1.71	0.947	0.086
Female	27	11	16	0.93 ± 1.62		
Age (years)						
<65	22	14	8	0.41 ± 1.23	0.051	3.799
≥65	80	30	50	1.07 ± 1.77		
Tumor size (cm)						
T < 5	50	23	27	0.92 ± 1.59	0.567	0.328
T ≥ 5	52	21	31	0.92 ± 1.79		
Differentiation						
Poor	91	35	56	1.04 ± 1.63	0.006	7.521
Moderate	11	9	2	−0.39 ± 1.90		
Lymphatic metastasis						
Absent	29	11	18	0.89 ± 1.92	0.829	0.047
Present	73	33	40	0.94 ± 1.59		
Venous invasion						
Absent	30	16	14	0.61 ± 1.82	0.180	1.801
Present	72	28	44	1.06 ± 1.62		
Perineural invasion						
Absent	25	17	8	0.26 ± 2.02	0.004	8.346
Present	77	27	50	1.14 ± 1.51		
Omentum majus invasion						
Absent	88	42	46	0.80 ± 1.63	0.040	4.228
Present	14	2	12	1.68 ± 1.85		
Histopathological typing						
Adenocarcinoma	89	37	52	0.94 ± 1.68	0.811	0.958
Signet-ring cell carcinoma	6	3	3	0.86 ± 1.41		
Others	7	4	3	0.74 ± 2.17		
Tumor location						
Cardia	40	17	23	1.16 ± 1.70	0.947	0.366
Antrum	23	9	14	0.67 ± 1.30		
Body	23	11	12	0.84 ± 1.93		
Others	16	7	9	0.80 ± 1.80		

that GC patients with a higher abundance of *P. intermedia* in their tumor tissues had a significantly shorter survival time (Figure 2B).

3.3 | The supernatant of *Prevotella intermedia* culture medium from tumor tissue promotes gastric cancer cell proliferation, migration, and invasion in vitro

To investigate the biological effects of *P. intermedia* on GC cells, we treated the GC cells with the supernatant of *P. intermedia* culture medium. Results indicated that tumor-derived *P. intermedia* (*P. intermedia*-T) had a more pronounced effect on promoting the growth of GC cell lines HGC-27 and MKN-45 compared to normal tissue-derived *P. intermedia* (*P. intermedia*-N; Figure 3A). Similarly, *P. intermedia*-T enhanced the cloning ability of GC cells (Figure 3B). Moreover, *P. intermedia*-T significantly accelerated the migration and invasion of GC cells when compared to *P. intermedia*-N (Figure 3C,D).

Further examination of genes related to epithelial–mesenchymal transition (EMT), proliferation, and apoptosis revealed noteworthy findings. After treatment with *P. intermedia*, the expression of N-cadherin, snail, bcl-2, and Ki-67 was upregulated, while E-cadherin and BAK1 expression were downregulated, possibly accounting for the observed changes in cell proliferation, migration, and invasion (Figure S1).

3.4 | Indirect co-cultivation with tumor-derived *Prevotella intermedia* alters the protein expression pattern of gastric cancer cells

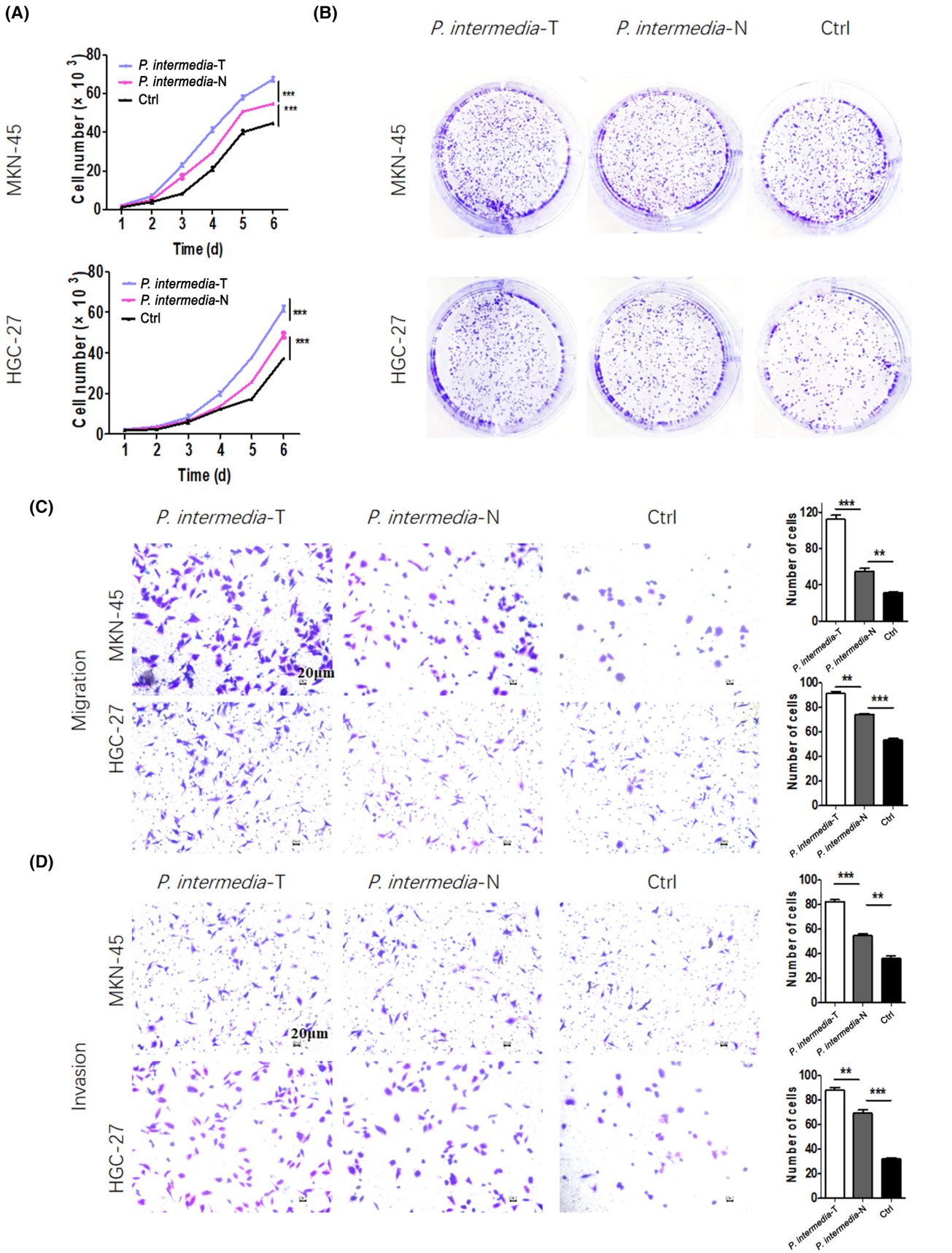
To delve deeper into the mechanism underlying *P. intermedia*-T's promotion of GC growth and metastasis, we used LC–MS/MS to identify changes in the protein profiles of GC cells following indirect co-cultivation with *P. intermedia*. A comprehensive analysis identified a total of 3354 proteins and 17,833 peptides. In the *P. intermedia*-T treatment group, 30 proteins exhibited increased expression, while 19 proteins showed decreased expression compared to the *P. intermedia*-N treatment group or the culture medium control (Figure 4A). These proteins were primarily associated with regulating cellular responses to organic substances, cellular component organization or biogenesis, cellular catabolism process, and macromolecular metabolism processes (Figure 4B).

To identify the unique molecular mechanism of *P. intermedia*-T's action on GC cells, distinct from the general characteristics of *P. intermedia*, we reanalyzed the distribution of these differential proteins using Venn diagrams. As a result, 34 proteins, including PLIN3, HSPA8, MYH9, GNB2L1, PARK7, ILF2, ARHGDIA, YBX1, TPT1, TPM4, PSMB1, PSMC3, ATL3, COPS2, SSB, MAT2A, SAFB, PSMD12, AHSA1, SSR4, DYNLL2, SRRM2, AIFM1, PRKCSH, PSAP, TMSB10, AGPS, CDK5RAP3, PREP, MCM2, MYDGF, GSTM3, UQCRFS1, and MKI67, were selected (Figure 4C). We analyzed the *Prevotella* abundance and the relative expression of the above genes in GC tissues in the TCMA database and found a correlation between the abundance of *Prevotella* and the relative expression of PLIN3 ($p = 0.005$, $r = 0.305$, Figure 4D). Subsequently, we analyzed the expression patterns and clinical significance of PLIN3 in the Cancer Genome Atlas (TCGA) database and found that PLIN3 was significantly overexpressed in GC tissues and related to a shorter survival time. However, there are few studies on the roles and potential applications of PLIN3 in GC. Therefore, quantitative PCR and western blot were performed to detect the expression of PLIN3 in GC cells treated with *P. intermedia*, confirming a significant increase in PLIN3 expression in the *P. intermedia*-treated group compared to the control group (Figure 4E, Figure S1b). These findings shed light on the potential role of PLIN3 in mediating the effects of *P. intermedia*-T on GC cells, providing valuable insights into the molecular pathways involved in GC growth and metastasis.

3.5 | Overexpression of perilipin 3 in gastric cancer and its effect on survival

Analysis of PLIN3 expression in GC, using data from the TCGA database, revealed a significantly higher expression in tumor tissues compared to paired healthy tissues (Figure 5A). Importantly, GC patients with elevated PLIN3 expression exhibited a shorter survival time (Figure 5B). To delve further into the role of PLIN3 in GC, we conducted knockdown experiments in GC cells (Figure 5C). Suppression of PLIN3 expression using shRNAs significantly inhibited the growth of GC cells (Figure 5D). In both MKN-45 and HGC-27 GC cell lines, knockdown of PLIN3 also resulted in reduced cell clone formation (Figure 5E). Similarly, inhibiting PLIN3 expression led to a notable decrease in the migration and invasion capabilities of GC cells (Figure 5F,G).

FIGURE 3 Biological effects of supernatant of *Prevotella intermedia* culture medium isolated from tumor tissue and matched healthy tissues. (A) Cell counting assay of gastric cancer (GC) cells treated with supernatant of *P. intermedia* culture medium isolated from tumor tissue and matched healthy tissues. $^{***}p < 0.001$. Control (Ctrl), using blank culture medium to treat GC cells. (B) Cell colony formation assay of GC cells treated with supernatant of *P. intermedia* culture medium isolated from tumor tissue and matched healthy tissues. Ctrl, using blank culture medium to treat GC cells. (C) Transwell migration of GC cells treated with supernatant of *P. intermedia* culture medium isolated from tumor tissue and matched healthy tissues, Magnification, ×200. Scale bar, 20 μm; $^{**}p < 0.01$, $^{***}p < 0.001$. Ctrl, using blank culture medium to treat GC cells. (D) Transwell invasion of GC cells treated with supernatant of *P. intermedia* culture medium isolated from tumor tissue and matched healthy tissues, Magnification, ×200. Scale bar, 20 μm; $^{**}p < 0.01$, $^{***}p < 0.001$. Ctrl, using blank culture medium to treat GC cells.



Furthermore, we investigated the effect of PLIN3 knockdown on the expression of key proteins associated with EMT, proliferation, and apoptosis. The results demonstrated that N-cadherin, snail, bcl-2, and Ki-67 expression levels were downregulated, while E-cadherin and BAK1 expression levels were upregulated upon PLIN3 knockdown (Figure S2).

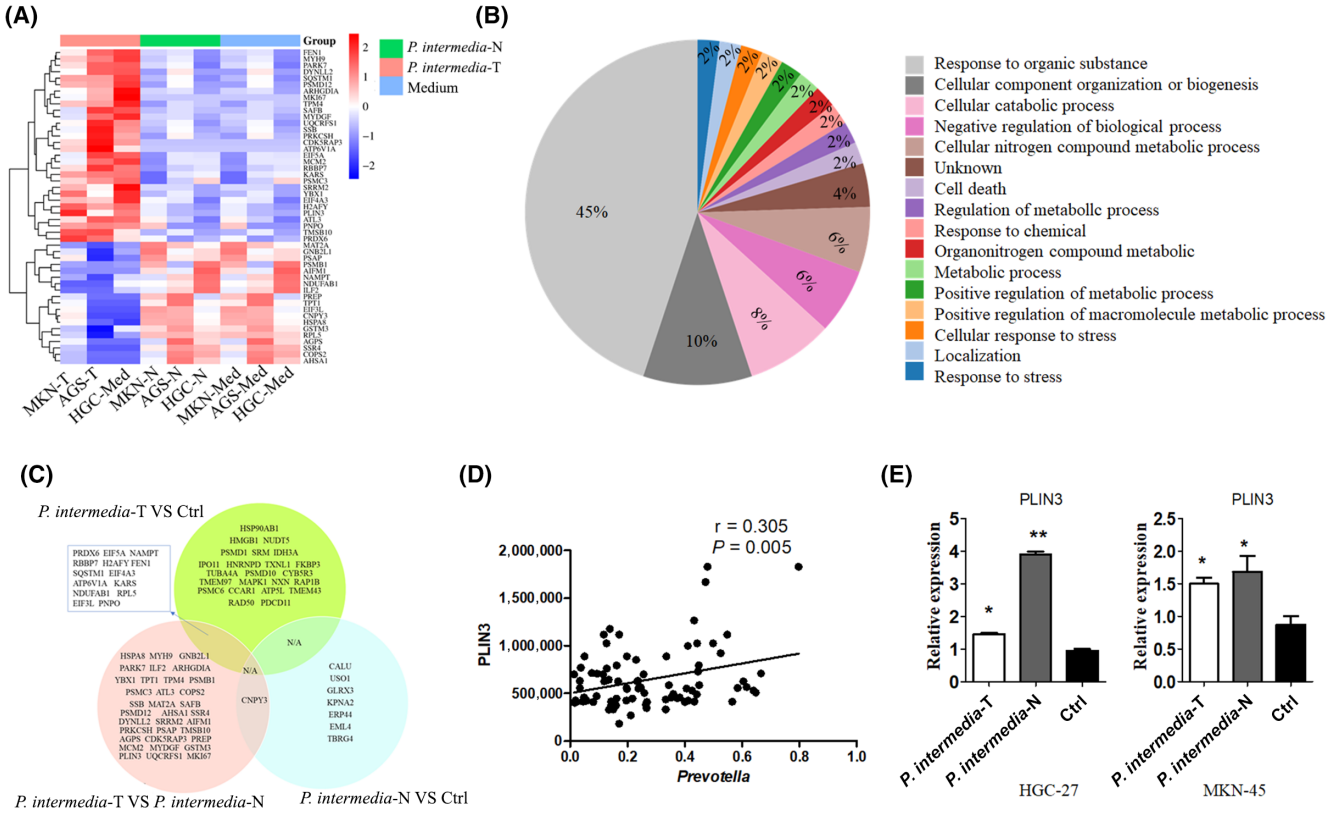


FIGURE 4 The altered protein expression pattern of gastric cancer (GC) cells treated with supernatant of *Prevotella intermedia* culture medium isolated from tumor tissue and matched healthy tissues. (A) Cluster graph of differential enrichment of proteins in GC cells treated with supernatant of *P. intermedia* culture medium isolated from tumor tissue and matched healthy tissues. (B) Biological function analysis of differential enrichment of proteins in GC cells treated with supernatant of *P. intermedia* culture medium isolated from tumor tissue and matched healthy tissues. (C) Venn diagram of differential enrichment of proteins in GC cells treated with supernatant of *P. intermedia* culture medium isolated from tumor tissue and matched healthy tissues. (D) Correlation analysis of *Prevotella* abundance and the relative expression levels of *PLIN3* in GC tissues. (E) The relative expression of *PLIN3* in GC cells treated with supernatant of *Prevotella intermedia* culture medium isolated from tumor tissue and matched healthy tissues.

3.6 | Inhibiting perilipin 3 expression diminishes the stimulating effect of tumor-derived *Prevotella intermedia* on gastric cancer cells

To investigate this further, we first knocked down PLIN3 before treating GC cells with *P. intermedia*-T, and the results showed a significant reduction in the proliferative effect of *P. intermedia*-T on GC cells (Figure 6A). Similarly, the promotion of clone formation in GC cells by *P. intermedia*-T was suppressed upon PLIN3 knockdown (Figure 6B). Furthermore, the migratory and invasive effects of *P. intermedia*-T on GC cells were significantly attenuated by shRNA targeting PLIN3 (Figure 6C,D). At the molecular level, the expression levels of N-cadherin, snail, bcl-2, and Ki-67 were downregulated, while E-cadherin and BAK1 expression were upregulated when PLIN3 was knocked down before *P. intermedia*-T treatment (Figure S3).

3.7 | *Prevotella intermedia*-T promotes xenograft growth depending on perilipin 3 in vivo

To further investigate the effect of *P. intermedia* on GC in vivo, we subcutaneously injected GC cells treated with *P. intermedia*-T into nude mice. The results indicated that *P. intermedia*-T was more effective in promoting the growth of xenografts than *P. intermedia*-N, and this effect was significantly weakened by PLIN3 knockdown (Figure 7A). The results of the weight analysis of tumors supported this finding (Figure 7B). H&E staining demonstrated that these xenografts were structurally similar, with tumor cells visible (Figure 7C). Immunohistochemical staining showed that Ki-67 and PLIN3 were upregulated when treated with *P. intermedia*-T and inhibited by PLIN3 knockdown (Figure 7D).

4 | DISCUSSION

Recently, there has been an increasing awareness that variations in microbial species or abundance in cancer patients play a crucial role in malignant tumor progression.[4,22] Notably, the gastric mucosa harbors a significant amount of viable symbiotic bacteria, and the bacterial load is markedly elevated in GC.[23] Apart from *H. pylori*, several other bacterial species have been isolated from GC tissues.[24] We successfully isolated and purified *P. intermedia* from GC tissues and paired healthy tissues.

Prior research has indicated that alterations in the intestinal microflora, such as a reduction in the growth of *Ruminococcaceae* and

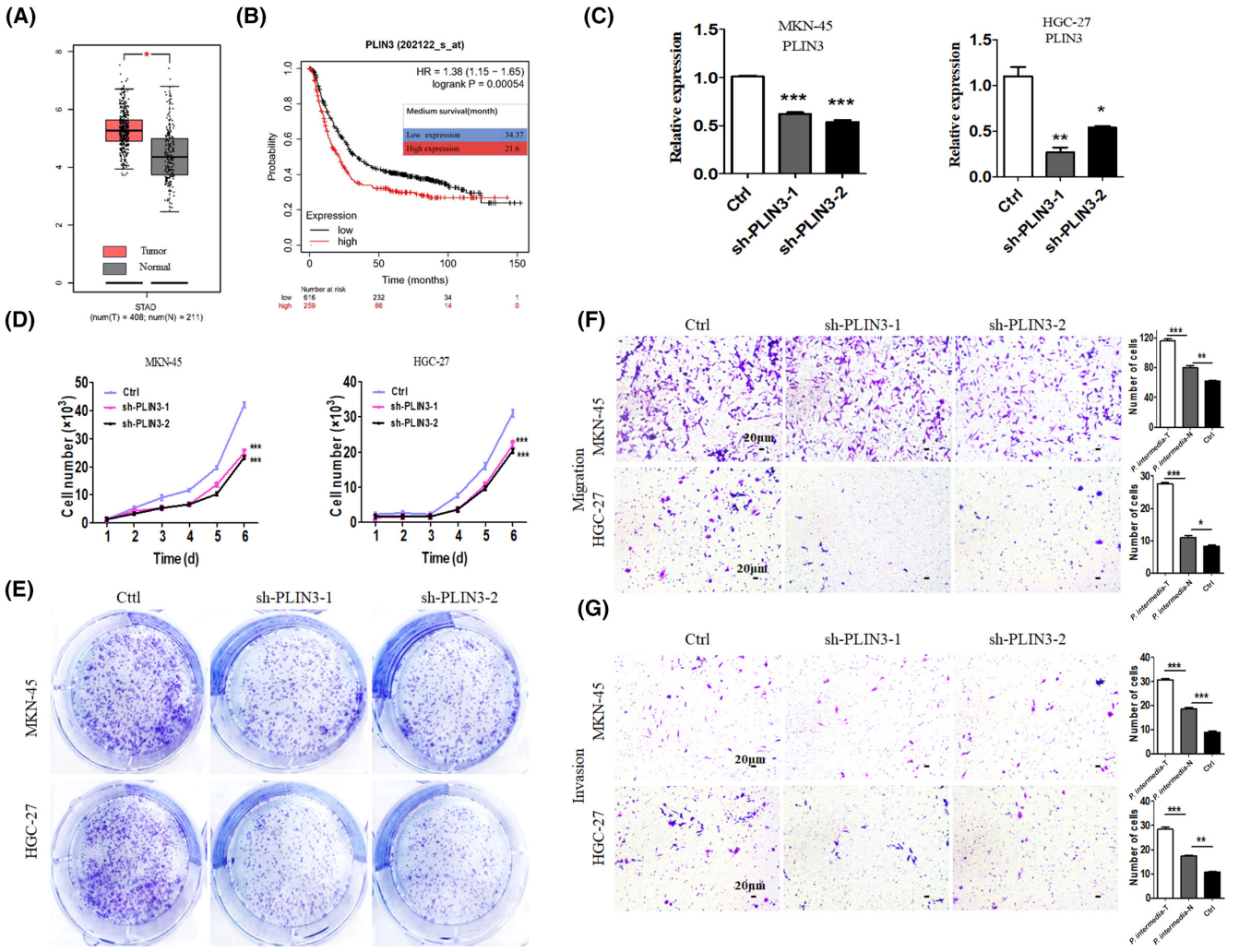


FIGURE 5 Biological effects of *PLIN3* in gastric cancer (GC) in vitro. (A) The relative expression of *PLIN3* in GC tissues; $^{*}p<0.05$. Data from https://portal.gdc.cancer.gov/. (B) The prognostic value of *PLIN3* in GC. Data from http://kmplot.com/analysis/. (C) The relative expression of *PLIN3* in GC cells using sh-*PLIN3*; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. (D) Cell counting assay of GC cells using sh-*PLIN3*, compared to control (Ctrl); $^{***}p<0.001$. (E) Cell colony formation assay of GC cells using sh-*PLIN3*. (F) Transwell migration of GC cells using sh-*PLIN3*. Magnification, ×200. Scale bar, 20 μm; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. (G) Transwell invasion of GC cells using sh-*PLIN3*. Magnification, ×200. Scale bar, 20 μm, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

Prevotellaceae flora, can inhibit GC formation.[25] We observed that *P. intermedia* was relatively enriched in GC tissues, and its presence correlated with a shorter survival time for GC patients in our study.

Studies on other bacteria have revealed their specific effects on GC. For instance, *Enterococcus faecalis* has been shown to induce the production of intracellular reactive oxygen species through an oxidative phosphorylation-independent pathway, resulting in restraint of DNA damage repair and downregulation of cell cycle regulation genes.[26] Another example is *Lactiplantibacillus plantarum YT013*, which was reported to inhibit GC cell proliferation and promote cell apoptosis through endogenous mitochondrial apoptosis pathways.[27] In this study, we focus on reporting the role of *P. intermedia* derived from GC tissues, which appears to promote cell proliferation, migration, and invasion in GC. These findings contribute to the growing understanding of the potential effect of microbial species on cancer progression. As *H. pylori* has been reported to play important roles in GC carcinogenesis, examining the interaction between *P. intermedia* and *H. pylori* in GC would further help to understand the mechanism of *P. intermedia* in GC. We analyzed the abundance of *Helicobacter* genus and *Prevotella* genus in GC tissues in the TCMA database and found a negative correlation between the abundance of *Helicobacter* genus and *Prevotella* genus (data from TCMA). Further research is vital for a deeper understanding of the interaction of *P. intermedia* and *H. pylori* in GC carcinogenesis.

As a gingival parasite, *P. intermedia* has primarily been reported as a predictive biomarker for periodontal disease and oral cancer.[28] In a case–control study based on biopsies, *P. intermedia* was detected in 83.3% (20/24) of patients with oropharyngeal squamous cell carcinoma via qPCR.[29] In our study, we made a significant observation: that the abundance of *P. intermedia* in GC tissue was notably higher than in matched healthy gastric mucosa tissue. Furthermore, our results revealed that the abundance of *P. intermedia* was associated

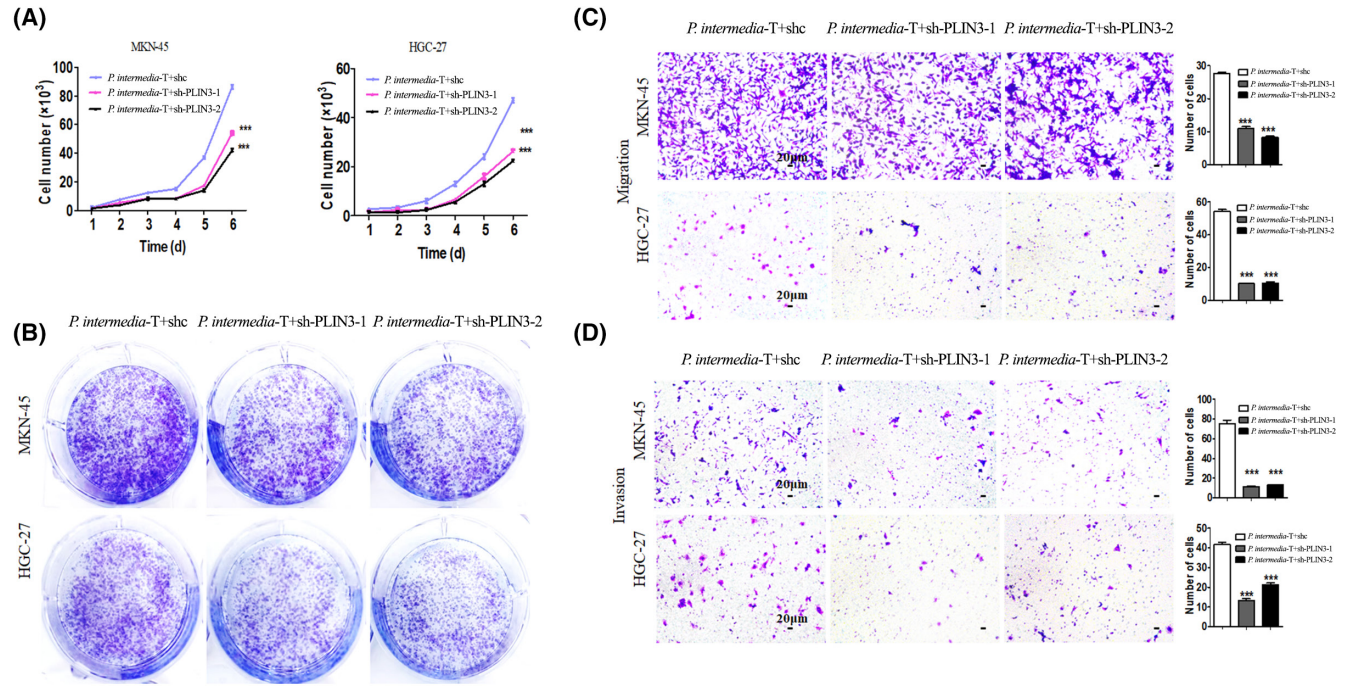


FIGURE 6 Biological effects of *PLIN3* in supernatant of *Prevotella intermedia* culture medium treated gastric cancer (GC) cells. (A) Cell counting assay of supernatant of *P. intermedia* culture medium-treated GC cells using sh-*PLIN3* compared to control (Ctrl); ***$p < 0.001$. (B) Cell colony formation assay of supernatant of *P. intermedia* culture medium-treated GC cells using sh-*PLIN3*. (C) Transwell migration of supernatant of *P. intermedia* culture medium treated GC cells using sh-*PLIN3*, Magnification, ×200. Scale bar, 20 μm; ***$P < 0.001$, compared with the group *P.int*-T + shc. (D) Transwell invasion of GC cells using sh-*PLIN3*. Magnification, ×200. Scale bar, 20 μm; ***$p < 0.001$, compared with the group *P.int*-T + shc.

with tumor differentiation, perineural invasion, omentum majus invasion, and the survival time of GC patients. Studies on the relationship between gastroenterological cancers and periodontitis showed a link between periodontal disease and increased risk of colorectal cancer (CRC).[30] It was also reported that periodontal disease was associated with a 52% increased risk of gastric adenocarcinoma.[31] However, another study on GC did not observe a significant association between the severity of chronic periodontitis and the risk of GC.[32]

Interestingly, a study by Lo et al. on CRC found that *P. intermedia* was significantly enriched in tumor tissue determined by 16S rRNA V3-V4 amplicon sequencing. Additionally, the secretome of *P. intermedia* (ATCC 25611) was shown to promote the proliferation and migration of CRC cells.[33] Our results indicated that the culture medium supernatant of *P. intermedia* isolated from tumor tissue had a promoting effect on GC cell proliferation, migration, and invasion.

Lipopolysaccharide derived from *P. intermedia* was shown to accelerate the release of tumor necrosis factor-alpha in macrophages transformed from monocytes, dependent on the mitogen-activated protein kinase signaling pathway.[34] Additionally, our study revealed that the secretome of *P. intermedia* promoted the expression of PLIN3 in GC cells. PLIN3 has been associated with the formation of lipid droplets in cancer cells, suggesting its potential involvement in fatty acid oxidation and tumor growth.[35] Consistent with this, our results indicated that PLIN3 was overexpressed in GC and played a role in promoting GC cell proliferation and metastasis. Furthermore, the data strongly suggested that tumor-derived *P. intermedia* exerted its effects on GC cell proliferation, migration, and invasion through its influence on the PLIN3 protein.

In a study of pancreatic cancer, another digestive system tumor, researchers identified 36 upregulated differentially expressed genes, including PLIN3, and 3 downregulated genes through bioinformatics analysis of 179 tumor tissue samples and 171 normal pancreatic tissue samples.[15] In vitro and in vivo investigations in prostate cancer cells indicated that depletion of PLIN3 led to decreased cell proliferation and enhanced radiosensitivity.[36] Similarly, in our study, knocking down PLIN3 in GC cells resulted in impaired cell proliferation, migration, and invasion. However, the role of PLIN3 in the therapeutic response of GC cells remains unclear. Additionally, we did not conduct in vivo experiments to demonstrate the role of PLIN3 in GC, representing one of the shortcomings of this study. Further investigations using animal models could provide valuable insights into PLIN3's effects on GC development and progression.

In conclusion, this study demonstrates that the heightened presence of *P. intermedia* is associated with tumor differentiation, perineural invasion, and omentum majus invasion, factors correlated with a shorter survival time in gastric cancer patients. These findings suggest that *P. intermedia* is a potential novel biomarker for gastric cancer. Moreover, *P. intermedia* derived from tumor tissue exhibited a more potent capability to promote gastric cancer cell proliferation and metastasis compared to that from healthy tissue, with this effect, in part, dependent on the protein PLIN3.

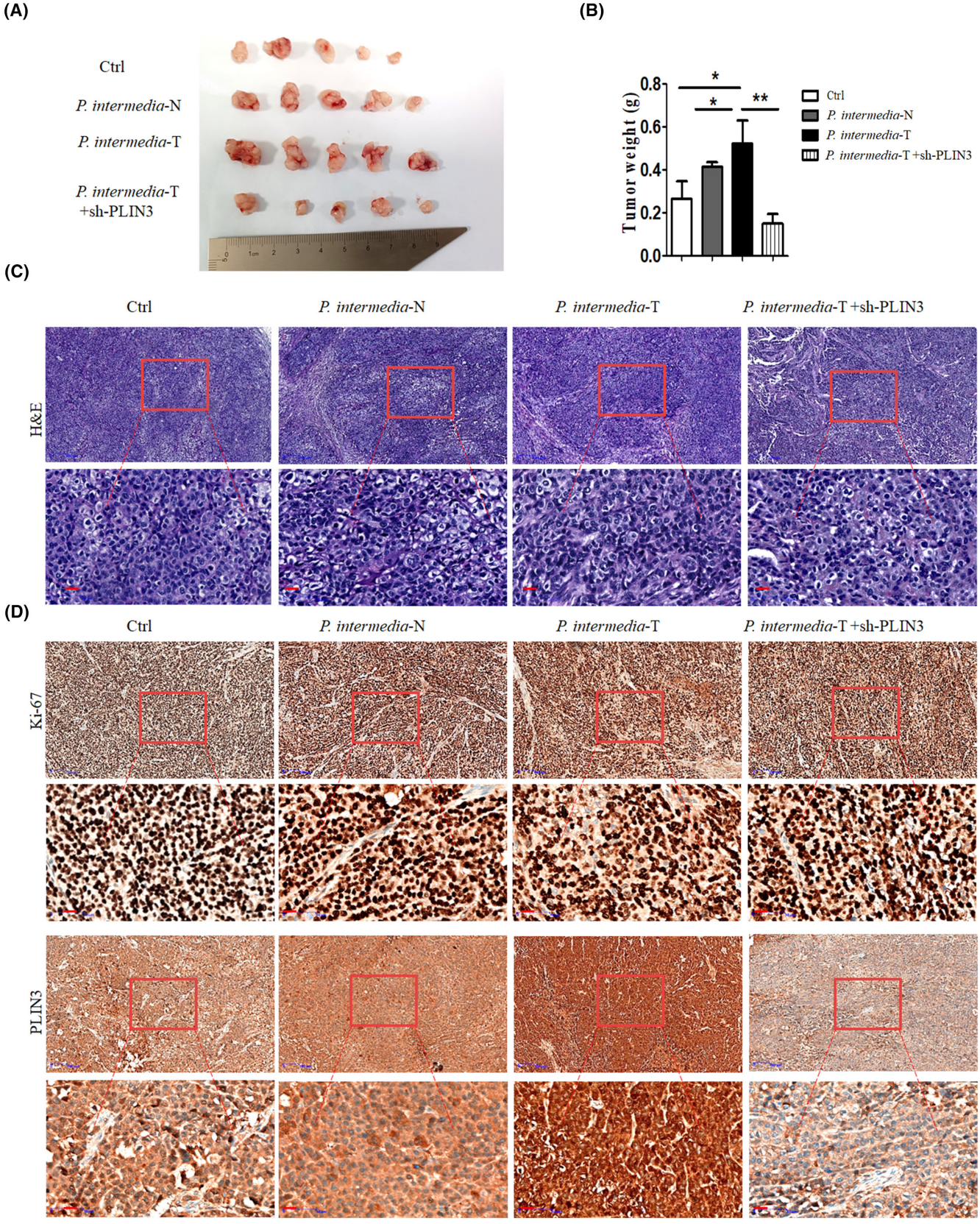


FIGURE 7 Effect of *Prevotella intermediate* on gastric cancer (GC) in vivo. (A) Photograph of tumors formed by the GC cells with different treatments. (B) The weight of the tumors formed by GC cells with different treatments; $^{*}p < 0.05$, $^{**}p < 0.01$. (C) H&E staining of xenograft. Magnification, ×200; scale bar, 20 μm. (D) Detection of Ki-67 and PLIN3 in tumors formed by GC cells with different treatment, Magnification, ×200; scale bar, 20 μm.

AUTHOR CONTRIBUTIONS

Wei Liang: Data curation; investigation; validation; visualization; writing – original draft. **Zhengyang Zhou:** Formal analysis; investigation; writing – review and editing. **Qizhao Gao:** Supervision; validation; writing – review and editing. **Zhichen Zhu:** Validation; visualization. **Jie Zhu:** Software; validation; writing – review and editing. **Jiayao Lin:** Methodology; software. **Yicheng Wen:** Formal analysis. **Feinan Qian:** Conceptualization. **Liang Wang:** Data curation. **Yaxuan Zhai:** Methodology; visualization. **Jingnan Lv:** Conceptualization; data curation; funding acquisition. **Haifang Zhang:** Funding acquisition; resources. **Fengyun Zhong:** Formal analysis; resources. **Hong Du:** Investigation; resources; supervision; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest for this article.

ETHICS STATEMENT

Approval of the research protocol by an institutional reviewer board: The study was carried out after approval by The Ethics Committee of The Second Affiliated Hospital on 2023–03–14. The number of Ethics Committee decisions is No. JD-LK2023007-101.

Informed Consent: N/A.

Registry and the Registration No. of the study/trial: N/A.

Animal Studies: N/A.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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