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Porphyromonas gingivalis detection by RNA *in situ* technologies: a reliable alternative to immunohistochemistry

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Abstract

To investigate whether the new RNA *in situ* hybridization test (RNAscope) could be a reliable alternative to immunohistochemistry (IHC) in detection of *Porphyromonas gingivalis* in esophageal squamous cell carcinoma (ESCC) tissues. The specificity of RNAscope probe was tested in esophageal cell lines infected by *P. gingivalis*. Thirty-five formalin-fixed, paraffin-embedded (FFPE) tissues from patients who were firstly diagnostic as ESCC in the First Affiliated Hospital of Henan University of Science and Technology were collected. *P. gingivalis* was detected by IHC and RNAscope using FFPE tissues. The ability and clinical prognostic value of RNAscope technique were analyzed and compared with that of IHC. RNAscope was specific for the detection of *P. gingivalis* in esophageal cells. Compared with IHC, the percentage of the sensitivity and specificity of RNAscope in detection of *P. gingivalis* in the cancerous tissues of ESCC patients were 92.9% and 95.2%, respectively. The

concordance rate was 94.3% between IHC and RNAscope (kappa = 0.881; $P < 0.001$). Importantly, the overall survival (OS) periods estimated by Kaplan–Meier analysis using the results of RNAscope or reference IHC test were similar (IHC, $P = 0.001$; RNAscope, $P = 0.004$). RNAscope is a novel and reliable method in detection of *P. gingivalis* and investigation of the pathogenesis of ESCC.

Keywords: Esophageal carcinoma, *Porphyromonas gingivalis*, RNA *in situ* hybridization, Immunohistochemistry

Introduction

Porphyromonas gingivalis, a gram-negative anaerobe, has been considered as a keystone pathogen in chronic periodontitis and other systemic diseases^[1]. Long-term colonization by *P. gingivalis* disrupts oral microecological homeostasis, influences and reshapes the host immune system, and alters host cell biological behavior and phenotype by modulating a range of signaling pathways^[2–4], which in turn affects tumor development^[5]. Studies have shown that *P. gingivalis* is enriched in the oral cavity and cancerous tissues of patients with esophageal cancer, which is associated with lower differentiation, greater invasiveness, and poorer prognosis of esophageal cancer^[6,7]. Thus far, there is no reliable and widely accepted techniques for detecting *P. gingivalis* in esophageal cancer. Immunohistochemistry (IHC) is currently a major technique used in detection of *P. gingivalis* in solid tissues such as esophagus and brain^[6,8–10]. However, several limitations of this technique prevent its extensive application in detection of infectious agents including *P. gingivalis*. These limitations include the highly antibody-dependent, non-standardized procedure and the unstable results caused by antibody specificity and subjective factors^[11]. In terms of *P. gingivalis* detection, the major issue using IHC is a lack of commercially available antibodies for *P. gingivalis*. Most of the antibodies reported in the literature were generated in-house^[6,10],

with high difficulty to access and lack of the information of specificity and sensitivity. This has greatly limited the study of *P. gingivalis* infection in esophageal cancer. Therefore, there is an urgent need for a new technique that is antibody-independent and can *in situ* display *P. gingivalis* in tissue.

RNAscope technology is a new generation of RNA *in situ* hybridization (ISH) technology developed in recent years^[12]. The technique is simple to use and is specific for the *in situ* visualization of target molecules in tissue samples (including paraffin-embedded tissue) due to its unique dual “Z” probe design principle and single molecule detection technique. Due to the unique sensitivity and specificity, RNAscope technology has been widely used in the field of gene expression of viruses^[13–16], bacteria^[17,18], and humans^[19,20]. Importantly, RNAscope has been used in the study of the genus *Fusobacterium* in colorectal carcinoma, specifically and clearly demonstrating the histological distribution of *F. nucleatum* in colorectal carcinoma *in situ* and metastatic carcinoma^[17]. Nevertheless, the use of RNAscope technology for the detection of *P. gingivalis* infection in esophageal cancer has not been reported.

In this study, we successfully established an mRNA ISH assay for *P. gingivalis* infection of esophageal cancer cells using the RNAscope probe. Moreover, we compared the strengths and limitations of IHC technique and RNA ISH in the detection of *P. gingivalis* infection in clinical samples of esophageal cancer. We believe that our findings will provide a reference for the application of the RNAscope technique in the study of esophageal cancer infection.

Materials and methods

Patients' information

Thirty-five cases of paraffin-embedded tissues were collected from the patients that were initially diagnosed and pathologically confirmed as esophageal squamous cell carcinoma (ESCC) in the First Affiliated Hospital of Henan University of Science and

Technology from January 2016 to December 2017. The surgically resected primary foci of ESCC and the corresponding Para-cancerous tissue specimens were harvested followed by formalin-fixed, paraffin-embedded (FFPE) and stored at room temperature. The pathological information was collected, and the esophageal cancer segmentation and TNM staging criteria were from the 8th edition of the TNM Staging Criteria for Esophageal Cancer. All patients signed informed consent before surgery and the study was approved by the Ethics Committee of the First Affiliated Hospital of Henan University of Science and Technology.

Experimental equipment and materials

P. gingivalis standard strain ATCC 33277 and whole bacterium antibodies were from Prof. Richard Lamont's lab (University of Louisville, USA). Human immortalized esophageal epithelial cell line NE3 was purchased from the Cell Bank of the Chinese Academy of Sciences. Hemoglobin chloride and vitamin K were purchased from Sigma (USA). Brain heart infusion (BHI) medium was purchased from BD, USA. Fetal bovine serum (FBS, USA) and RPIM-1640 medium were purchased from Gibco and Life Technologies, USA. 8-well EZ slides were purchased from Mitsubishi (Germany). RNAscope® PPIB positive control probe (item no. 313901), dapB negative control probe (item no. 310043-C2), RNAscope® 2.5HD kit (red, item no. 322350) and HybeZ hybridization oven were purchased from Advanced Cell Diagnostics (ACD). The immunohistochemistry kit and DAB chromogen were purchased from Beijing ZhongShan Jinqiao Biotechnology Co.

Cell and bacterial culture

P. gingivalis standard strain ATCC 33277 was cultured in sterile BHI medium containing 1% hemoglobin chloride and 0.1% vitamin K, and in an anaerobic incubator at 37°C (85% N₂, 10% H₂ and 5% CO₂) for 24h. The bacterial concentration is measured

by spectrophotometer at 600 nm and the OD600 value is usually up to 1.0~1.5 after 24-hour culture. Resuscitated NE3 cells were cultured in RPIM-1640 liquid medium with 10% fetal bovine serum at 37 °C in a 5% CO₂ incubator.

Infection of NE3 cells with *P. gingivalis*

To test the specificity of the RNAscope probe for detection of *P. gingivalis*, a gradient of different concentrations of *P. gingivalis* was set up to infect NE3 cells by taking *P. gingivalis* bacterial solution at logarithmic growth stage, adjusting the bacterial solution concentration to 1×10^8 copies · mL⁻¹ according to the absorbance value of bacterial culture medium at 600 nm, followed by taking 1 mL of bacterial solution, centrifuging the supernatant at 12000 rpm for 5 min at 4°C, and resuspending with 1 mL of sterile PBS. Then, count the cells and infect them at a ratio of 10:1 and 100:1 (i.e. multiplicity of infection, MOI 10, 100) for 6h.

Immunohistochemistry

Paraffin-embedded tissue from the above esophageal carcinoma was cut into 4 μm thick continuous paraffin sections followed by dewaxing and antigen repair using microwave-heated 0.01 mol · L⁻¹ citrate buffer and immunohistochemistry SP with rabbit anti-human *P. gingivalis* polyclonal antibody at a concentration of 1:1000, and 0.01 mol · L⁻¹ PBS (pH = 7.5) instead of primary antibody, which was used as a negative control. Positive expression of *P. gingivalis* was determined by the presence of brownish-yellow color in the tissue cells. Refer to literature for specific interpretation methods and scoring criteria [6].

Probe design and synthesis

Based on the GenBank sequence of the bacterial genome of *P. gingivalis* ATCC 33277 (NC_010729.1), 20 pairs of specific probes, named FirmA, were designed, and synthesized by ACD (item no. 300031-

C2; sequence protected by patent and not available).

P. gingivalis detection by RNAscope *in situ* hybridization

Sample preparation for cell and tissue sections

NE3 cells were plated and grew in 8-well slides and divided into 2 groups. The first group of NE3 cells was used as a blank control and the second one was infected with *P. gingivalis*. Then, the cells were fixed 24 h after infection. Samples were prepared for subsequent experiments according to the instructions in the RNAscope user the manual “Preparation and Pre-treatment of Adherent Cell Samples” provided by the manufacturer. Cell infection experiments were repeated three times independently.

Serial sections of paraffin-embedded tissue from esophageal cancer using 4 μm as described above were baked for 1 hour in a 60°C drying oven for dewaxing. RNAscope target repair and Proteinase Plus treatment were carried out under standard conditions and subsequent steps were carried out according to the instructions in Formalin Fixed Embedding (FFPE) Sample Preparation and Pre-treatment.

In situ hybridisation and interpretation of results

Probe hybridization: The procedure was carried out according to the RNAscope® 2.5HD Assay Kit (Red) User Manual. Three consecutive sections of each sample were hybridized for *P. gingivalis*, PPIB and dapB probes. The PPIB probe is used to assess the amount of hybridisable RNA in the sample and to determine the adequacy of RNA in the sample when the majority of tissue cells in the section show a significant hybridization signal. The dapB probe is used for quality control non-specific hybridization staining and the sample is only suitable for RNAscope hybridization if the dapB probe is negative, or if the hybridization signal is extremely weak.

Interpretation of results: It was performed independently by two pathologists. Hybridization results were observed on a standard bright-field microscope (Nikon ECLIPSE 80i) at 20 $\times$ magnification, with small spots in the tissue/cells visible in positive con-

trols at 20–40 $\times$ magnification, while in negative controls, there are no staining spots or <1 signal spot per 10 cells in each field of view.

Based on the study of *F. nucleatum*^[17], the signal evaluation criteria for *P. gingivalis* target probes were set as below. For each sample, 10 fields of view were randomly selected under a 40 $\times$ objective and the number of *P. gingivalis* staining spots per 10 cells was counted. The results were interpreted using the following criteria: 0 point for no signal, 1 point for 1–10 spots, 2 points for 11–30 spots, 3 points for 31–60 spots and 4 points for > 60 spots. A score of ≥ 1 is considered positive for *P. gingivalis*.

Statistical analysis

In this study, the statistical significance of difference between count data, which exhibits in the value of percentages (%), was evaluated by Chi-square test. For count data and paired samples was analyzed by student's t-test and analysis of variance (ANOVA). All analyses were performed SPSS 19.0. Cohen's Kappa measure was used to assess methodological consistency. Survival time was acquired from the regular follow-up phone-calls and analyzed by the one-way Kaplan-Meier method and a Log-Rank test. $P < 0.05$ was considered as a statistically significant difference.

Results

Analysis of clinical data and detection of *P. gingivalis* with immunohistochemical

Thirty-five FFPE tissues were included in the study. The median age of the tested ESCC patients was 62 years (range 46–78 years), with 68.6% ($n = 24$) male and 31.4% ($n = 11$) female. Most of the tumors occurred in the middle and lower esophagus and the overall lymph node metastasis rate was 28.6% (10/35). The clinical TNM stage was mainly stage I + II. Pathological information of the ESCC patients is presented in Table 1. A positive immunohistochemical signal for *P. gingivalis* was 40.0 % (14/35).

Table 1. Association between the status of *P. gingivalis* infection and the clinicopathologic features of ESCC patients

Factors (No.)	Results by IHC		<i>P</i> value
	Negative	Positive	
ESCC samples (35)	21 (60.0%)	14 (40.0%)	
Gender			
Male (24)	14 (58.3%)	10 (41.7%)	0.766
Female (11)	7 (63.6%)	4 (36.4%)	
Age, years			
≤ 60 (15)	9 (60.0%)	6 (40.0%)	1
> 60 (20)	12 (60.0%)	8 (40.0%)	
Tumor location			
Upper (5)	4 (80.0%)	1 (20.0%)	0.592
Middle (13)	7 (53.8%)	6 (46.2%)	
Lower (17)	10 (58.8%)	7 (41.2%)	
Differentiation			
Well (6)	3 (50.0%)	3 (50.0%)	0.462
Moderate (12)	6 (50.0%)	6 (50.0%)	
Poor (17)	12 (70.6%)	5 (29.4%)	
Lymph node metastasis			
Positive (10)	7 (70.0%)	3 (30.0%)	0.704
Negative (25)	14 (56.0%)	11 (44.0%)	
TNM stage			
I + II (24)	14 (58.3%)	10 (41.7%)	0.766
III (11)	7 (63.6%)	5 (36.4%)	

RNA-ISH assay specifically detects *P. gingivalis* in cultured cells

To verify the feasibility of RNAscope for *P. gingivalis* assay, we first performed ISH on NE3 cells with the specific probe in the presence or absence of *P. gingivalis* infection. Cells were infected with a serial of doses of *P. gingivalis* (multiplicity of infection: 10, 100) followed by the ISH assay. We found that *P. gingivalis* (red dots) was detected in the cell lines with a dose-dependent signal and nonvisible background, and no signals were detected for the use of negative control (dapB) (Figure 1).

RNA-ISH assay specifically detects *P. gingivalis* in ESCC tissues

RNAscope for *P. gingivalis* test was carried out on the tissue sections from 35 ESCC patients. Overall, fourteen (40.0%) cases were positive for *P. gingivalis* RNA-ISH. Of these samples, 12 (85.7%) cases had 1 point, 1 (7.1%) case had 2 points, and 1 (7.1%) case had 3 points. *P. gingivalis* was observed in the tumor center and adjacent mucosa cells of positive cases. In contrast, no positive signals were detected in normal tissue (Figure 2). These results therefore showed the invasive *P. gingivalis* distribution was highly heterogeneous and focal, which could be in the cytoplasm, or invade the glandular structure near the edge of the tumor.

Comparison of IHC and RNA-ISH in the detection of *P. gingivalis*

We next analyzed the concordance between the expression of *P. gingivalis* whole antigens and mRNA, as well as the validity of RNAscope technology in tissues. Of the 35 whole tissue sections, 14 cases were *P. gingivalis* positive in IHC assay, 14 positives in RNAscope assay. Compared with IHC, the sensitivity and specificity of RNAscope technology were 92.9% and 95.2%, respectively. The concordance rate was 94.3% (kappa = 0.881; $P < 0.001$) between the IHC and RNAscope assays (Table 2).

Comparison of IHC and RNA-ISH in predicting the overall survival rate of ESCC

A total of 35 patients were followed up for survival analysis over a period of 65 months. The mean survival time for patients with positive *P. gingivalis* antigen expression was 31.0 months, which was significantly lower than that of *P. gingivalis* negative group (49.0 months) ($P = 0.001$). Similar results were found when mRNA expression was examined (both $P < 0.05$). Kaplan-Meier analysis showed that OS was strongly correlated with RNAscope and IHC (Figure 3).

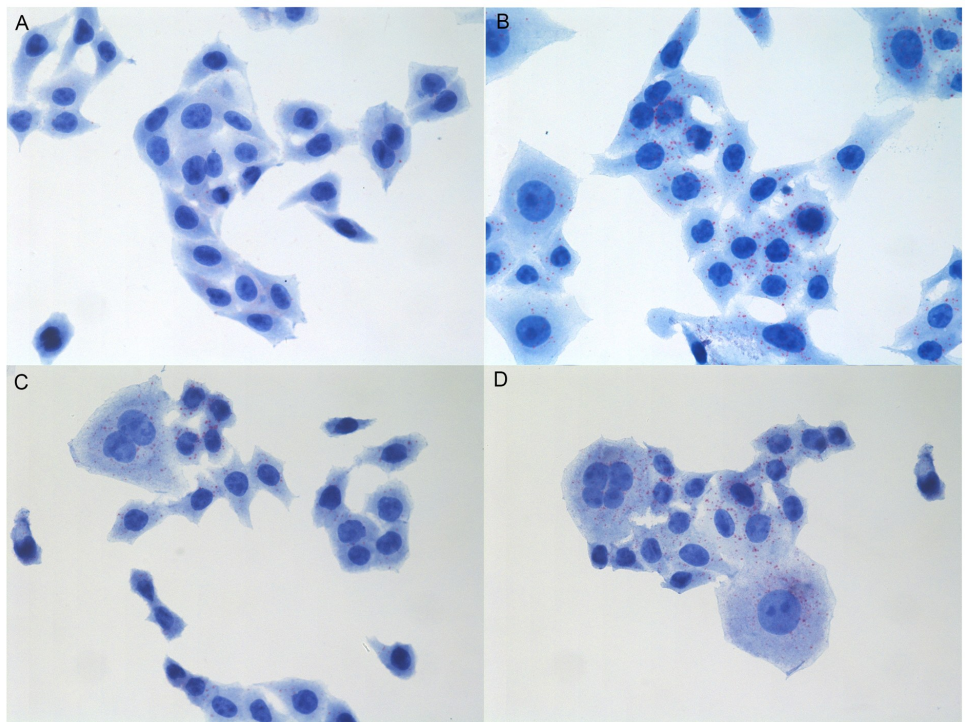


Fig 1. RNAscope detects the presence of *P. gingivalis* in NE3 cell lines. NE3 cells were detected with (A) dapB probe (negative) and (B) PPIB probe (positive); *P. gingivalis*-infected NE3 were detected with (C) FirmA probe (MOI 10) and (D) FirmA probe (MOI 100). 40×magnification.

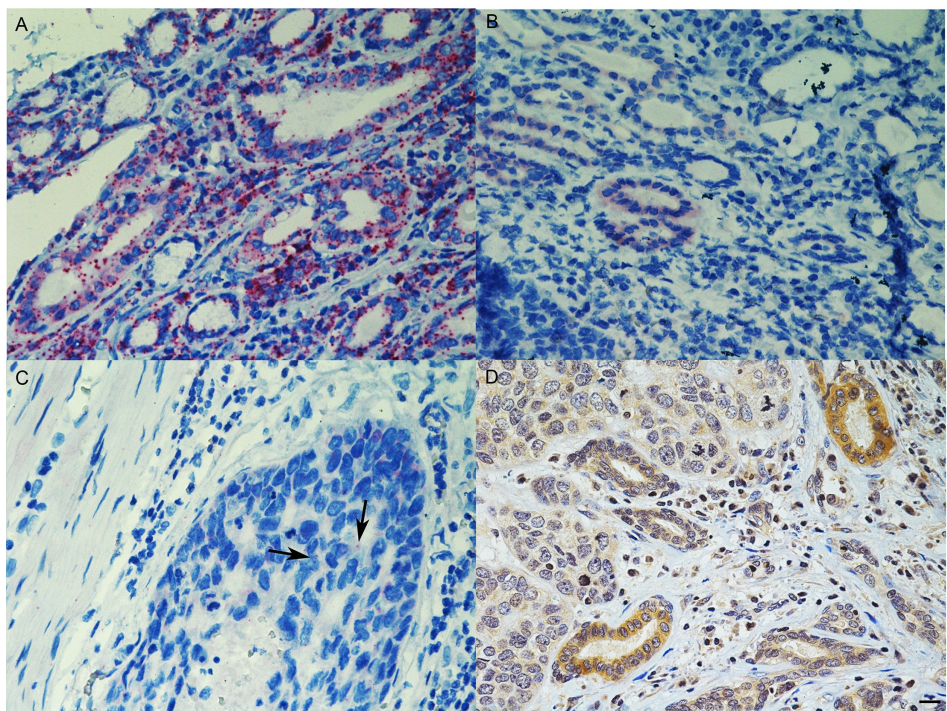


Fig 2. RNAscope detects the presence of *P. gingivalis* in ESCC tissues. (A) Representative images of PPIB positive control probe in esophageal cancer tissue (red spots); (B) Esophageal tumor tissue stained with dapB negative control probe; (C) Representative images of *P. gingivalis* distribution stained by specific probe (red spots indicated by black arrows); (D) The same FFPE tissue stained by immunohistochemistry. scale bar = 50 μ m.

Table 2. Concordance between *P. gingivalis* whole cell antibodies and mRNA expression in the cancerous tissue from patients with ESCC

Approaches	RNAscope		Sensitivity, %	Specificity, %	Kappa	P
	Positive	Negative				
IHC(+)	13	1	92.9	95.2	0.881	< 0.001
IHC(-)	1	20				

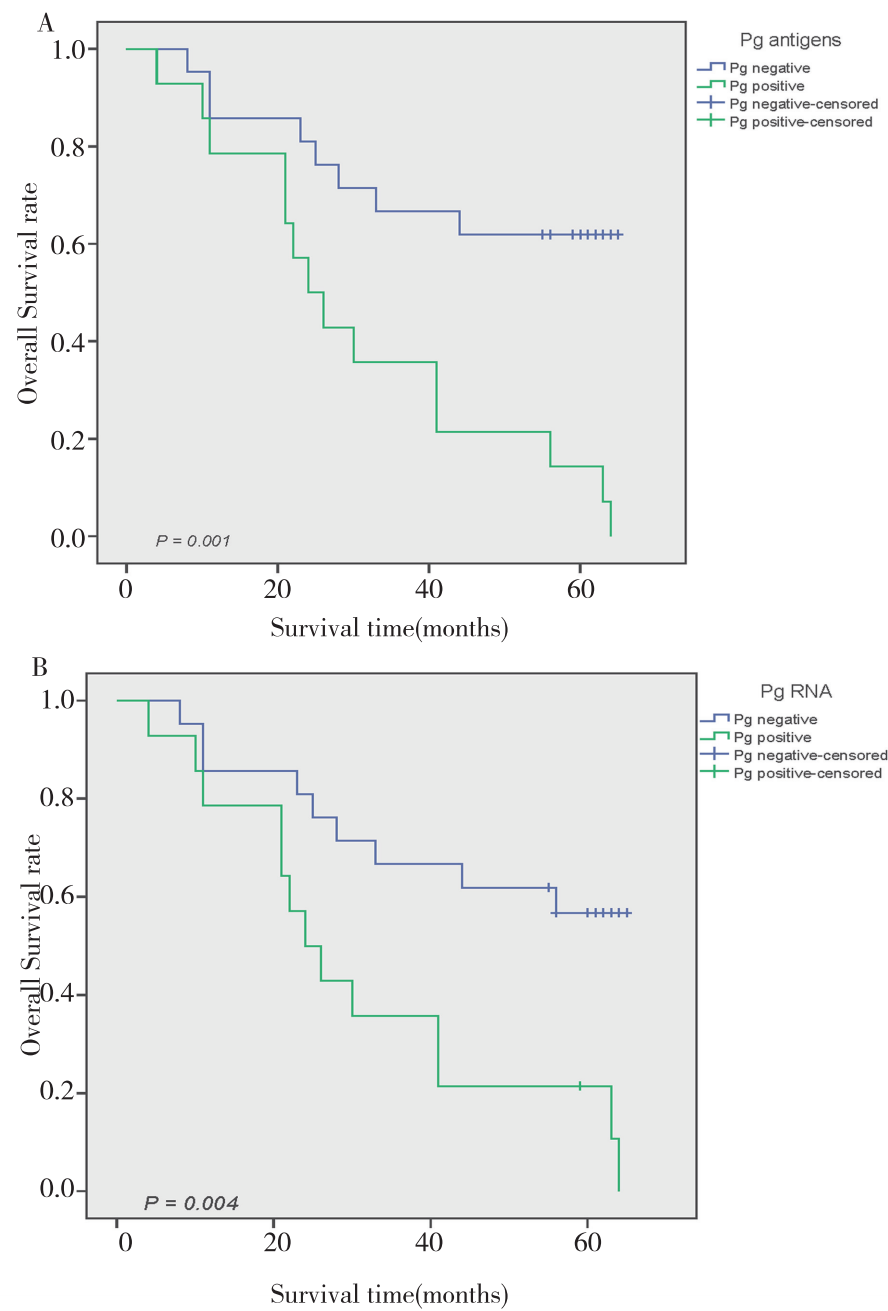


Fig 3. Overall survival rate of patients with ESCC derived from the analysis of the results of IHC and RNAscope. (A) *P. gingivalis* antigens expression by IHC ; (B) *P. gingivalis* mRNA expression by RNAscope.

Discussion

P. gingivalis has been considered as a keystone pathogen in chronic periodontitis, as well as a potential etiological agent for the development of oral, esophageal, and other cancers^[21,22]. The possible mechanisms by which *P. gingivalis* is involved in the onset or exacerbation of diseases has been widely studied^[23]. *P. gingivalis* infection was found to promote the development of esophageal cancer by increasing the expression of B7-H4 to assist tumor cells immune escape^[24]. Moreover, intracellular invasion of *P. gingivalis* also promotes the proliferation and migration of ESCC through the miR-194/PTEN/Akt signals^[25].

We have examined the presence of *P. gingivalis* in ESCC tissues by IHC and demonstrated that the whole cell antigens of *P. gingivalis* were primarily expressed in the epithelial cytoplasm of cancerous and adjacent esophageal mucosa, but occasionally in nuclei^[6]. In this study, the similar distribution of *P. gingivalis* mRNA was found by RNA in situ hybridization. Additionally, a large number of *P. gingivalis* also formed a biofilm-like structure, which was observed in the space of esophageal cancerous cells. In contrast, no signals were observed in adjacent non-carcinomatous tissues. These findings therefore suggest the ability of *P. gingivalis* to invade cancerous cells *in vivo* and confirmed that *P. gingivalis* can invade and colonize cancerous cells, proliferate, and survive in the cytoplasm of infected tumor cells, and then spread to surrounding cells^[26].

RNA-scope, one of the newest ISH methods, is a highly sensitive and specific test. The ability to detect mRNA signals using standard microscopy makes it a powerful tool in detection of pathogen infection^[27]. Using multiple probes, Victoria B et al. analyzed the FFPE sections from a range of tumor types with mRNA ISH and the results indicate that ISH can successfully evaluate mRNA in prospectively and retrospectively collected tissue samples^[11]. Recently, many studies on SARS-CoV-2 analyses using RNA-

scope have improved its compatibility with other technologies, and further expanded its application^[13,28]. Here, we utilized RNA-scope to detect ESCC cells infected with different concentration of *P. gingivalis*. Our findings showed that the hybridization signal significantly increased with the increased amount of *P. gingivalis*, which verified its technical characteristics, low background, and specificity.

Despite of the discrepancy in 2 of the cases, in our study the sensitivity and specificity of RNA-scope assay are well consistent with that of IHC in detection of *P. gingivalis* both for cultured cell and tumoral tissues. Notably, among these cases, 1 was positive in IHC and negative in RNA-scope assay, which may be resulted from the existence of *P. gingivalis* antigens and no transcriptional activity genes. In addition, another 1 case with *P. gingivalis* mRNA positive were negative in IHC assay (1 score), which may be from mistaken interpretation of IHC results. Nevertheless, the inconsistencies between RNA-scope and IHC are also happened in recent HPV studies^[14,29]. On the other hand, the overall survival of *P. gingivalis*-positive patients with ESCC was significantly better than their *P. gingivalis*-negative counterparts regardless of the definition of *P. gingivalis*-status (IHC, RNA-scope). Therefore, it was verified again that the prognosis of ESCC patients with *P. gingivalis* infection was poor.

In summary, *P. gingivalis*-RNA-scope showed a high specificity and can *in situ* detect the distribution of *P. gingivalis* in cells and tissues. The potential value of RNA-scope in predicting the prognosis of ESCC was equivalent to the IHC assay. Therefore, it is an effective technique to detect infectious agents, especially *P. gingivalis*, in esophageal cancer and thus could be a reliable alternative to immunohistochemistry in the assay of gene *in situ* expression.

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Competing interests

The authors declare that they have no competing interests in this study.

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