Cancer Letters 404 (2017) 1–7

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Article

Different frequencies of *Porphyromonas gingivalis* infection in cancers of the upper digestive tract

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ARTICLE INFO

Article history:
Received 20 April 2017
Received in revised form
13 June 2017
Accepted 4 July 2017

Keywords:
Porphyromonas gingivalis
Upper digestive tract cancer
16S rDNA
Acid tolerance
Mix infection
Synergistic pathogenicity

ABSTRACT

The high incidence rate of multiple carcinomas in the upper digestive tract is often explained in terms of involvement of the same underlying risk factors. It has been reported that the oral bacterium *Streptococcus anginosus* is associated with esophageal, gastric, and pharyngeal cancers. We previously reported occurrence of *Porphyromonas gingivalis (P. gingivalis)* DNA in esophagus cancer. In this study, the presence of *P. gingivalis* in specimens of various types of cancer from the upper digestive tract was investigated. Here we report that *P. gingivalis* was preferentially and frequently present in specimens of esophageal cancer as well as in those from dysplasia of the esophagus but rarely in matched noncancerous portions and are quite low or absent in cancers from the cardia or stomach. Therefore, it led us to propose that, the microorganism does not survive in conditions of high acidity. We then investigate the pH dependence of survival of *P. gingivalis* as well as the acid tolerance of it. We found that, exposure to acidic buffers of a wide range of pH values led to a decline in colony forming units of *P. gingivalis*, thus, providing a possible explanation for variations in frequencies of *P. gingivalis* infection in this study.

Introduction

Microbes are among the environmental factors that may contribute to the etiology of cancer [1,8]. Given the link between *Helicobacter pylori* and gastric cancer [11], the presence of microorganisms in several other kinds of gastrointestinal (GI) cancers was also widely investigated, while *Streptococcus anginosus (S. anginosus)* DNA fragments were frequently found in cancerous samples from esophagus [15,16,21], stomach [20], as well as head and neck squamous cell carcinomas [22,24]. However, the constitution and diversity of the microbiota in different sections of the upper GI tract under health and various disease states, as well as the function of microbiota in the pathogenesis of various digestive diseases are still undefined.

Porphyromonas gingivalis (*P. gingivalis*) is a common gram negative anaerobic oral bacterium strongly associated with periodontal disease [9]. Although this bacterium has been proven to invade gingival epithelial cells [9], studies on presence of the bacteria at the epithelium from different sites of upper digestive tract are very scarce. Previously, we had reported the occurrence of *P. gingivalis* DNA fragments in surgical specimens of Esophageal squamous cell carcinoma (ESCC) [7], leading us to propose that *P. gingivalis* infection occurs in esophagus cancer tissues as well as other sites in the upper digestive tract. The purpose of the present

Abbreviations: ESCC, Esophageal squamous cell carcinoma; *P. gingivalis*, *Porphyromonas gingivalis*; *F. nucleatum*, *Fusobacterium nucleatum*; *S. anginosus*, *Streptococcus anginosus*; GI, gastrointestinal; CFU, colony-forming units; IHC, Immunohistochemistry.

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http://dx.doi.org/10.1016/j.canlet.2017.07.003
0304-3835/

study was, therefore, to investigate the frequency of the presence of *P. gingivalis* DNA in cancerous tissues from esophagus, cardia and stomach by IHC and qPCR analysis.

Here we report the preferential infection of *P. gingivalis* in esophageal cancers, much less frequently in cancerous tissues of the cardia and was absent from the gastric cancer tissues. The distribution of *P. gingivalis* DNA led us to propose that the organism does not survive in conditions of high acidity. We then went to investigate how pH affects the growth and survival of *P. gingivalis in vitro*, in order to provide a possible explanation for variations in frequencies of *P. gingivalis* infection in cancers of the upper digestive tract.

Materials and methods

Bacterium strain

P. gingivalis strain American Type Culture Collection (ATCC) 33277 and W83 were grown on anaerobic blood agar plates in a chamber containing 85% nitrogen, 5% hydrogen, and 10% carbon dioxide for 3–5 days. It was then inoculated into brain-heart infusion broth for 24 h until the culture reached an optical density of 1.0 (OD600), corresponding to-10^8 colony-forming units (CFU)/ml. Culture *P. gingivalis* and culture medium were diluted with saline in the same dilution before experiment. Bacterial strains were maintained as frozen stocks and after being started were passed three times through this growth medium before being used in the experiments.

Study subjects

From 2010 to 2014, a multicenter research of esophageal cancer drew subjects primarily from the Taihang Mountain region of Henan provinces where ESCC occurs at very high rates, including the First Affiliated Hospital of Henan University of Science and Technology (HUST; Luoyang, Henan, China) and Anyang Tumor Hospital (ATH; Anyang, Henan, China). Adjacent tissue samples were obtained 3 cm distant to cancerous tissue. Thirty additional specimens were randomly selected during endoscopic examination from biopsy, and confirmed histologically as normal esophagus mucosa. Thirty-three cardiac cancer tissues, twenty-five gastric cancer tissues and their adjacent non-cancerous tissues were obtained from patients at ATH. The institutional review boards at HUST and ATH reviewed and approved this study, and all participants signed written sheets of informed consent.

No restrictions regarding age, sex, or disease stage were set. Patients who had received any preoperative radiotherapy, chemotherapy or immunotherapy therapy before recruitment or any blood transfusion in the preceding 6 months or antibiotics consumption in the preceding 6 days were excluded. All examined esophageal cancers were squamous cell carcinomas (ESCC) and all examined gastric cancers (GC) were adenocarcinomas. Cardiac cancers were located in the proximal 3 cm of the stomach, while non-cardiac cancers were those in the remainder of the stomach. The clinical stage and histological tumor type of ESCC were determined according to the UICC/AJCC TNM Classification of 2009 (seventh edition). Patient's clinical information was collected from the medical records of the patients and stored in a database, which was updated every 3 months by telephone follow-up. Complete follow-up was updated until death or January 2015. The specimens were collected and treated promptly after surgery. Each specimen was sufficient to be cut into three pieces and treated differently for various uses, put in liquid nitrogen for extracting RNA, or fixed in 10% formaldehyde for making paraffin embedded blocks, or grinded sterilely and anaerobically for isolation and identification of *P. gingivalis* clinical strains from ESCC lesions.

Immunohistochemistry (IHC)

Tissues were fixed in formalin and then embedded in paraffin. Serial sections of 4 mm thickness were prepared and deparaffinized by submersion in three separate concentrations of ethanol (100, 95, and 70%), and rinsing continuously in distilled water for 5 min. Antigen retrieval was performed by incubating slides in antigen retrieval Citra plus solution (BioGenex, San Ramon, USA), according to the manufacturer's instructions. Slides were blocked 1.5% normal goat serum (Vector Laboratories, Burlingame, USA) for 30 min. Polyclonal rabbit anti-*P. gingivalis* 33277 [26] was utilized for the detection of *P. gingivalis*. Pre-immune rabbit IgG and normal mouse IgG was used as a negative control. Primary antibodies were incubated with tissue sections (1:1000 dilution) for 12 h, 4 °C, followed by biotin-conjugated secondary antibody for 1 h at room temperature, streptavidin-peroxidase for 30 min at room temperature, and enzyme substrate (3,3′-Diaminobenzidine, Dako, Denmark). As an additional control, sections were also incubated with phosphate buffered saline (PBS) only, followed by incubation with biotin-conjugated secondary antibody, streptavidin-peroxidase, and enzyme substrate. PBS washes (3 times, 5 min each) were performed during each incubation step. Sections were counterstained with methyl green and visualized by light microscopy (Eclipse 80i, Nikon, Japan). Every tissue section was evaluated by two senior pathologists. Staining intensity was classified using a numerical scale; grade 0 (none, 0–10% staining); grade 1 (weak, 10–30%); grade 2 (moderate, 30–60%), and grade 3 (strong, over 60%), with a score of ≥2 considered positive of staining with *P. gingivalis*.

Determination of 16S rDNA in cancerous tissues

Tissues were suspended in 500 μl of sterile phosphate-buffered saline, vortexed for 30 s and sonicated for 10 s. Proteinase K (2.5 mg/ml final concentration) was added and the samples were incubated overnight at 55 °C, homogenized with sterile disposable pestle and vortexed. DNA was extracted as described previously [7] and purified by phenol-chloroform extraction. All samples were stored at −80 °C until further analysis. PCR for amplification of 16S rDNA of *P. gingivalis* was performed in a total volume of 25 μl containing 2 μM of primers and 10 ng of template DNA. 16S rDNA samples were amplified as described previously [7] using *P. gingivalis* specific and universal 16S rDNA primers (*P. gingivalis* 16S rDNA primer sequences were: 5′AGGCAGCTTGCCATACTGCG3′ (forward) and 5′ ACTGTTAGCAACTACCGATGT 3′ (reverse), and the PCR product size was 404 bp; The universal 16S rDNA primer sequences were 5′GATTAGATACCCTGGTAGTCCAC3′(forward) and 5′CCCGGGAACGTATTCACCG3′ (reverse), and the PCR product size was 688 bp. The PCR cycling conditions were 30 cycles of denaturation at 94 °C for 30 s, annealing at 65 °C for 30 s with a decrease of 0.2 °C per cycle, and extension at 72 °C for 30 s.

Acid survival assay

P. gingivalis strains were grown overnight, harvested, and washed once with 20 mM phosphate-buffered saline (PBS; pH 7.0). The acid challenge buffer was made fresh that day and the range of pH of the buffer used was between pH 2.5 and 8.5. The pH of the buffer between 4.5 and 8.5 was achieved by mixing various amounts of 100 mM sodium phosphate monobasic and 100 mM sodium phosphate dibasic to the desired pH. Below a pH of 4.5 the desired pH was achieved by the addition of HCl. The pH of the buffer during the course of the experiment did not change by more than 0.1 pH units. Cells were suspended in the buffer at a concentration of 2×10^8 CFU/ml and were incubated for a period from 15 to 60 min. The viable *P. gingivalis* colonies were counted after 4–5 days, and the results were expressed as CFU/ml.

Statistical analysis

All statistical analyses were performed by SPSS statistical package, version 17.0 (SPSS Inc., Chicago, IL, USA). Correlations between the presence of *P. gingivalis* in the adjacent, cancerous tissues and normal esophagus mucosa were analyzed by Chi-square test; Correlations between the presence of *P. gingivalis* in the cancerous tissues from upper digestive tract were analyzed by Chi-square test; The agreement between the two different methods of IHC and qPCR was analyzed by Chi-square test; Correlations between the presence of mixed infection (*P. gingivalis* and *F. nucleatum*) and clinicopathologic factors were analyzed by Chi-square test, as appropriate. P values of ≤0.05 were considered to be statistically significant.

Results

Quantities of P. gingivalis DNA in non-cancerous tissue samples of esophagus

By qPCR analysis using specific primers, for detection of *P. gingivalis*, DNA fragment was amplified in twenty-four of fifty (48%) esophageal cancers (Fig. S1), whereas only one of thirty (3.3%) of the corresponding noncancerous portion of the esophagus showed positive signals ($\chi^2 = 17.412$ $P < 0.001$), as presented in Table 1.

P. gingivalis is detected in the specimens of dysplasia of esophagus

Squamous cell carcinoma in the esophagus is thought to arise through the dysplasia-carcinoma sequence, thus, we also

Table 1
Presence of *P. gingivalis* in normal esophagus mucosa, cancerous and adjacent tissues of ESCC.

Tissues	n	No.(%) of positive samples	
		IHC	*qPCR*
[1]Esophagus Cancer	50	21 (42%)	24 (48%)
[2]Dysplasia of esophagus	30	6 (20%)	7 (23.3%)
[3]Normal esophageal mucosa	30	1 (3.3%)	1 (3.3%)

IHC:1,2: $\chi^2 = 4.059$ $P = 0.044$; 1,3: $\chi^2 = 14.061$ $P < 0.001$; 2,3: $\chi^2 = 2.588$ $P = 0.108$.
PCR: 1,2: $\chi^2 = 4.807$ $P = 0.028$; 1,3: $\chi^2 = 17.412$ $P < 0.001$; 2,3: $\chi^2 = 5.192$ $P = 0.023$.