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Prevalence of multiple infections and the risk of gastric adenocarcinoma development at earlier age

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ABSTRACT

Helicobacter pylori and Epstein–Barr virus are well established infections for gastric cancer development. However, the role of cytomegalovirus alone or in combination with other infections is unclear. In this case-control study, the prevalence of different infections was evaluated, and their frequency was compared with clinicopathologic features among gastric cancer patients and normal volunteers from 2012 to 2017. Approximately two-thirds (61.9%) of the gastric cancer patients had at least 1 viral infection, while viral infection prevalence in normal volunteers was only 4.7% ($P = 0.021$). The higher infection frequency in gastric cancer patients was observed for EBV (49.2%). No CMV DNA was detected in normal volunteers. In contrast, one-fourth of the gastric cancer patients were infected with CMV. Furthermore, CMV frequency in tumoral tissues (68.75%) was significantly higher than in nontumoral tissues (12.5%) ($P = 0.0311$). Although *H. pylori* infection was significantly lower in tumoral tissues than in nontumoral tissues ($P = 0.0136$), all tumoral tissues had cagA, while only 61.5% of nontumoral tissues were cagA positive. CMV-infected patients were affected 14 years earlier than uninfected, and CMV-negative patients (mean age = 56 vs. 69 and 70 years; $P = 7.6\times10^{-3}$ and $P = 2.7\times10^{-4}$, respectively). Also, EBV viral load in earlier grades and stages was more than 100-fold higher than advanced grades and stages. Our results show a high level of infections in gastric cancer. The association of these infections especially with CMV contributes to gastric adenocarcinoma development at earlier age.

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1. Introduction

Gastric cancer (GC) is among the commonest causes of cancer-related deaths worldwide. Although the incidence of GC has decreased in developed countries during the last 5 decades, there is still a significant mortality rate associated with GC (Crew and Neugut, 2006; McCullough et al., 2001). GC has high incidence in Eastern Asia, Eastern Europe, and South America, while low incidence was observed in North America and Europe (Torre et al., 2015). The development of GC occurs as a result of genetic alterations, and infectious and other environmental agents' interference (Gonzalez et al., 2002; Saghier et al., 2013). The infectious agents can increase the risk of cancer via integration of their genomes into the host's chromosomes, long-term inflammation in a part of the body, or immune system inhibition (Coussens and Werb, 2002; Finlay and McFadden, 2006; Yu et al., 2005). Relatively, according to The Cancer Genome Atlas research, Epstein–Barr virus (EBV)– associated GC is 1 of the 4 subtypes of a new molecular classification of GC (Anon., 2014). EBV is also associated with a number of other human malignancies, including diffuse nasopharyngeal carcinoma, Hodgkin's disease, and Burkitt lymphoma (Brady et al., 2007; Flavell and Murray, 2000; Young and Dawson, 2014). *Helicobacter pylori* (*H. pylori*) is another pathogen that colonizes the stomach. Chronic infection with *H. pylori* strains harboring cagA gene can increase the risk of site-specific diseases, such as gastritis, peptic ulcers, mucosa-associated lymphoid tissue lymphoma, and gastric adenocarcinoma (Wroblewski

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et al., 2010). *H. pylori* shows higher infection rate in developing countries than in developed countries. In addition to bacteria and EBV, several other viruses are also known as potential environmental triggers for the cancer. Accordingly, about 12% of all cancers around the world have been estimated to be caused by viruses (Tashiro and Brenner, 2017). Human cytomegalovirus (HCMV) is 1 of the 8 known viruses in the herpes family that chronically infects about 50–90% of the world's population (Herbein and Kumar, 2014). HCMV is considered as an oncomodulator virus that infects tumor cells and increases their malignancy (Michaelis et al., 2009). Although there is a controversy about its role in cancer development, numerous evidence supported the pivotal role of HCMV in many cancers including malignant glioma, prostate, skin, breast, and colorectal cancers (Chen and Chan, 2014; Cobbs et al., 2002; Herbein and Kumar, 2014). Hepatitis B virus (HBV) that may cause both acute and chronic diseases is another well-known virus that acts as a major risk factor for hepatocellular carcinoma. Although HBV is considered to be a hepatotropic virus, several studies have reported the presence of HBV in nonliver tissues including pancreas, skin, vessel walls, bone marrow, colon, lymph nodes, and kidneys (Dejean et al., 1984; Ma et al., 2011). HBV was also linked to non-Hodgkin's lymphoma and pancreatic cancer (Lim et al., 2007). Moreover, serological analysis showed an association between hepatitis B surface antigen, and GC (Wei et al., 2015). As yet, however, there is little evidence about the prevalence of HBV DNA in GC tissues. Based on these observations and considering the importance of using antiviral and antimicrobial agents in treating cancer patients in combination with traditional cancer therapies, we aimed to investigate the prevalence of infectious agents in GC, as well as determine the clinicopathological features of GC patients with infections.

2. Methods

2.1. Subjects

A total of 126 samples derived from 63 patients (63 tumoral tissues and their adjacent nontumoral counterparts), who had been diagnosed for gastric adenocarcinoma and had not undergone previous chemotherapy, were enrolled in the study. Samples were collected from primary tumors of patients who underwent surgery at different hospitals in Mazandaran province, Iran, from January 2012 through June 2017. Fresh surgical samples including tumoral and their adjacent nontumoral tissues were snap frozen in liquid nitrogen and stored at −80 °C for subsequent analyzes. Adjacent nontumoral tissues were obtained from the histologically nontumoral tissue at a distance of at least 5 cm from the tumor. Twenty-one healthy individuals who underwent elective esophagogastroduodenoscopy were selected as normal controls. All subjects gave their written consent before sample collection. This study was performed in accordance with the guidelines of ethics committee of Babol University of Medical Sciences.

2.2. Pathological analysis

A part of collected samples was fixed in 10% formalin, sliced into tiny segments, and embedded in paraffin. The paraffin-embedded specimens were sliced into 5-μm sections and stained with hematoxylin and eosin. The pathological stage and grade of the tumor, as well as the histological feature of the nontumoral tissue, were determined by a pathologist according to the American Joint Committee on Cancer criteria.

2.3. Detection and quantification of pathogens

DNA was extracted from normal tissues, tumoral tissue, and tissues adjacent to tumor using the QIAamp DNA mini Kit according to the manufacturer's instructions. The quality and quantity of the obtained DNA were assessed using NanoDrop 2000 (Thermo Scientific, USA). Real-time polymerase chain reaction (PCR) for virus detection was performed on a Stepone plus real-time PCR system (Applied Biosystems, Life Technologies, USA) by HotStarTaq Plus DNA Polymerase (Qiagen, Hilden, Germany) in the presence of specific primers and probe that amplify segments including the C gene of HBV, UL83 gene of HCMV, and BamH1W gene of EBV (Khansarinejad et al., 2012; Leb et al., 2004; Ryan et al., 2009). PCR conditions for all primers and probe were 95 °C for 5 min, followed by 50 cycles of 15 s at 95 °C and 1 min at 60 °C. PCR-based diagnosis of *H. pylori* was carried out using ureC and cagA genes specific primers described previously (Lage et al., 1995). For viral load quantification and cells copy number determination, PCR products were cloned into pMD18 vector, and then the number of template copies was estimated from the plasmid concentration and the molecular weight of each plasmid molecule. The human *ABL* gene was used to validate the efficiency of DNA isolation and to estimate the copy number of pathogens per cell in a given volume of extracted DNA. The resulting ratio was used to calculate the number of copies of EBV and CMV per 50,000 cells. The formula for making this conversion is as follows:

$$\text{Copy number} = \frac{\left(\text{amount of amplicon (ng)} \times 6.022 \times 10^{23}\right)}{\left(\text{length of dsDNA amplicon} \times 650 \times 10^{9}\right.}$$

2.4. Statistical analysis

Chi-square and *t* test were used to determine differences between groups. P value < 0.05 was considered as statistically significant

3. Results

3.1. Clinicopathological characteristics

The clinical characteristics of the enrolled patients are illustrated in Table 1. The median age of the patients was 68 years (range, 36–89 years), and the male-to-female ratio was 4:1. Overall, 65% of tumors were located at proximal position of the stomach (cardia, fundus, and body) and 35% at distal position (antrum and pylorus). Highly and poorly differentiated tumors were observed in 18% and 82% of patients, respectively. As shown in Table 1, according to TNM classification, most samples were in stage II with a metastasis to lymph nodes. Indeed, 12.7 % of patients had a positive family history of cancer.

3.2. H. pylori infection

Overall, *H. pylori* DNA was detected in 29/63 (46%) of GC specimens. Among the 29 *H. pylori*–positive cases, in 10 cases, both tumoral and the adjacent nontumoral tissues were infected. In 16 cases, only the adjacent nontumoral tissues were infected, and in 3 cases, only the tumoral tissues were infected. Interestingly, the prevalence of *H. pylori* infection

Table 1
Patients' clinicopathological characteristics.

Characteristics		%
Gender	Male	80.9
	Female	19.1
Age	<68 y	49.2
	≥68 y	51.8
Differentiation	Well differentiated	18
	Moderately/poor differentiated	82
Stage	I	21.8
	II	41.9
	III	30.9
	IV	5.4
Lymph node metastasis	Yes	66.6
	No	33.4

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