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Human Papillomavirus (*HPV*) Prevalence and E6 Protein Expression in Gastric Cancer Tissue Samples Compared with Non-malignant and Control Groups in East Azerbaijan Province, Iran, 2021

Abolfazl Jafari-Sales[1], Afsoon Shariat[1]*, Hossein Bannazadeh-Baghi[2], Behzad Baradaran[3], Behboud Jafari[4]

1. *Department of Microbiology, School of Basic Sciences, Kazerun Branch, Islamic Azad University, Kazerun, Iran*
2. *Department of Virology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran*
3. *Department of Immunology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran*
4. *Department of Microbiology, School of Basic Sciences, Ahar Branch, Islamic Azad University, Ahar, Iran*

ABSTRACT

Background and Aim: Gastric cancer (GC) is a major health problem worldwide. Several studies have shown the virus's role in cancer pathogenesis. Therefore, the aim of this study was to evaluate the presence of *human papillomavirus* (*HPV*) in GC by immunohistochemistry (IHC) and polymerase chain reaction (PCR) in hospitals in the East Azerbaijan province, Iran.

Materials and Methods: In this descriptive cross-sectional study, 100 tissue samples of paraffin-embedded, including GC (50 samples), benign gastric hyperplasia (25 samples), and a control group (25 samples) were collected from the archives of laboratories in East Azerbaijan province from April to October 2021. The IHC and PCR were used to detect the *HPV* virus. SPSS software version 22 and t-test and Chi-Square statistical tests were used for data analysis.

Results: 8 out of 50 cancer samples were *HPV* positive by IHC and PCR. *HPV*-positive samples had a mean age of 62.87 ± 9.67. Men had the highest number of *HPV*-positive samples compared to women (5 samples vs. 3 samples). However, no viral genomes were observed in non-malignant and control samples. There was a significant relationship between *HPV* infection and GC (*P*=0.03).

Conclusion: According to the present study's findings, the presence of *HPV* infection in GC plays an important role in the development of GC in the hospitals of East Azerbaijan province. Also, the results showed that PCR and IHC are both more sensitive and reliable in detecting *HPV* because the results of both IHC and PCR were the same. Hence IHC method can be used as an alternative to the PCR method to detect *HPV* oncoproteins.

Keywords: E6 protein, Gastric cancer, *Human papillomavirus*, Immunohistochemistry, PCR

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Corresponding Information: **Afsoon Shariat**, *Department of Microbiology, School of Basic Sciences, Kazerun Branch, Islamic Azad University, Kazerun, Iran* Email: afsoonsh1980@yahoo.com

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1. Introduction

Gastrointestinal carcinomas are a group of cancers characterized by a high risk of occurrence and a relatively high mortality rate. Gastric cancer (GC) is the third leading cause of cancer death in both sexes worldwide (1). The prevalence of this disease is higher in the male population in developing countries (456,000 in men and 221,000 in women), East Asia, South America, and Eastern Europe (2). In addition, the incidence of GC varies in different geographical areas. High-risk areas include Japan, Korea, China, Chile, Costa Rica, Brazil, and medium-risk areas include Italy, the United Kingdom, Germany,

Netherlands, Turkey, Iran, and low-risk areas include the United States, Canada, Sweden, Denmark, Egypt, India and Australia **(3, 4)**. In Iran, unlike developed countries, the incidence of GC is increasing. This increase is more in the west of Iran than in other places **(5, 6)**.

The pathogenesis of GC is multifactorial. Improper eating habits are one of the important etiological factors in the incidence of GC. In addition, smoking and alcohol consumption have a significant effect on the growth of GC. Chronic infection with *Helicobacter pylori* (*H. pylori*) or *Epstein-Barr virus (EBV*) also plays an important role in the development of GC **(7)**.

Neoplastic changes are a long, complex, multi-step process that various genetic and epigenetic changes can cause. Various studies show that virus infection can directly or indirectly lead to multiple malignant tumors **(8, 9)**.

The *human papillomavirus* (*HPV*) is a non-enveloped, double-stranded, relatively small virus with a genome of 8,000 bp, which is a suspected risk factor for neoplastic deformity. There is ample evidence of carcinogenic properties of *HPV* in studies of anal cancers **(10)**, oral **(11)**, throat **(12)**, ovarian **(13)**, cervical **(14)**, and breast **(15)**, which indicate the role of the virus in the pathogenesis of cancer in other parts of the gastrointestinal tract. The major viral proteins that cause carcinogenesis are E6 and E7, which bind to and degrade p53 and Rb, respectively, thus preventing apoptosis and enhancing cell cycle progression **(16)**. E6 protein with 150 amino acids is a nuclear protein that migrates to the nucleus after being made in the cytoplasm by the NLS sequence. E6 is a transforming protein **(16)**. So, continued expression of E6 and E7 is required to produce invasive cancer with a malignant phenotype, which generally occurs after years of persistent *HPV* infection **(17)**. Currently, more than 150 *HPV* subtypes have been identified with 15 species of high-risk types of *HPV* (HR-*HPV*) **(18)**. *HPV16* and *HPV18* are the most common subtypes of *HPV* in cancer worldwide, which are associated with tumors of the gastrointestinal tract, such as cancer of the mouth, esophagus, and colon **(19)**. In Iran, the rate of GC varies according to the level of risk (high, moderate, or low). The northern and northwestern regions are high-risk areas for GC, while other geographical areas are moderate and low-risk **(4)**.

Possible environmental risk factors have been suggested as the reason for this change **(3)**. The prevalence of *HPV* in Iran is almost the same as in Germany and Spain **(20)**. In Iran, the prevalence of *HPV* in gastrointestinal cancers has been reported between 34.2% and 49.5%, respectively **(21-23)**. The prevalence of *HPV* in patients with GC in China is 31% **(24)**, in Brazil 10% **(25)**, and in the Netherlands 0% **(26)**. These studies show different evidence of the association between gastric cancer risk and viral infection in different regions. But so far, no research has been done in East Azerbaijan province. Also, due to the high rate of *HPV*-positive tumors, reliable diagnostic techniques should be used to improve the diagnosis result for *HPV*-positive patients and improve the survival rate. The aim of this study was to investigate the presence of *HPV* DNA and E6 viral protein expression in GC tissue samples compared with non-malignant and control groups in East Azerbaijan province using polymerase chain reaction (PCR) and immunohistochemistry (IHC). If *HPV* infection is confirmed in GC patients, these patients also need viral infection treatment.

2. Materials and Methods

Sample Collection

In this descriptive cross-sectional study, 100 formalin-fixed, paraffin-embedded tissue samples were collected from the pathology archive of the pathology group, clinical center, and hospitals of East Azerbaijan province (Ghazi Tabatabaee, Sheykh Al-Raees, Aalinasab, Imamreza and Razi Hospital), Iran, from April to October 2021. They were retrospectively selected as follows: 50 samples from patients with GC, 25 non-malignant samples (gastric ulcer (GU)), and 25 normal controls (healthy adjacent to the tumor samples).

The pathologist histologically approved tissue samples. Cancer patients included 31 (62%) men and 19 (38%) women. Inclusion criteria included blocks from patients with GC and benign gastric hyperplasia whose paraffin blocks had sufficient tissue for IHC and complete information on age, sex, and histopathological grade. Exclusion criteria included uninformed blocks and previous anticancer treatment. Clinical characteristics and follow-up results were also obtained from the electronic medical record. The clinical and histopathological characteristics of the patients included in this study are presented in Table 1. The present study was conducted in accordance with the guidelines of the Helsinki Declaration and its subsequent amendments, and informed consent was obtained from all patients.

Preparation of Samples for IHC

From 100 samples of paraffin blocks, sections with a thickness of 5 to 10 µm were created using a microtome device (Leitz, Germany) and collected on sterile slides. To prevent cross-contamination, sharp blades were used, and gloves were replaced between each sample cut. According to the protocol of PAKGENE kit (Iran) for paraffin removal the samples, the slides were exposed

to xylene (1 mL) and ethanol (96%) and watered to different degrees. Samples were incubated using a 3% hydrogen peroxide solution and methanol to suppress internal peroxidase activity. Nonspecific bands with normal goat serum will be inactivated after rinsing with phosphate buffer. Samples were subjected to specific IHC staining using Mouse / Rabbit PolyDetector HRP / DAB C1P5 made by Daco Denmark. Monoclonal antibodies and diaminobenzidine were stained as chromogenic material by the Streptavidin biotin peroxidase technique **(27)**. Using a light microscope (Olympus Corporation, Tokyo, Japan), slides were evaluated for E6 viral protein expression. The nucleus of cancer cells and sometimes the cytoplasm have pigmented deposits indicating the presence of viral proteins. Two pathologists evaluated IHC results with the same opinion.

Table 1. Clinical and histopathological characteristics of the studied groups

Attributes		n		
		GC (n=50)	Non-malignant (n=25)	Control (n=25)
Gender	male	31	16	15
	female	19	9	10
Age		61.7±12.01	58.04 ± 13.63	63.88 ± 16.05
Grade	G1	1	-	-
	G2	14	-	-
	G3	35	-	-
Staging	T1	3	-	-
	T2	6	-	-
	T3	27	-	-
	T4	14	-	-

DNA Extraction

Isolation of genomic DNA from paraffin blocks was performed using a kit made by PAKGENE Iran according to its protocol. The extracted DNA was purified with 50 µL of Tris-EDTA, and the DNA concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc. Waltham, MA, United States). Briefly, 200 µL of the sample was digested with 20 µL of proteinase K and 200 µL of AL buffer at 56°C for 10 minutes. DNA precipitation was performed by adding 200 µL of 96% ethanol. The DNA was washed in 200 µL of AE buffer, and the resulting DNA was stored at -20°C until further testing **(27)**.

PCR Reaction to Detect *HPV*18

PCR reaction was performed using DNA extracted from tissue samples for the presence of *HPV* and *HPV18* by general and specific primers. According to Table 2, the primers that had the maximum specificity were selected. The reaction was adjusted to a volume of 25 µL, including 12.5 µL of Master mix, 10 pmol of each primer, 3 µL of DNA, and 8 µL of deionized water. The PCR temperature program for *HPV* infection using primers of the *HPV18* genotype was as follows: initial denaturation at 94°C for 6 min, followed by 35 cycles including denaturation at 94ºC for 50 s, annealing at 57ºC to for 50 seconds, extension/elongation was performed at 72°C for 55 seconds and final expansion at 72°C for 6 minutes. Genomic DNA of HeLa cell line containing *HPV18* was prepared and used as a positive control by Tabriz Immunology Research Center. Negative control included PCR reaction mixtures without the addition of template DNA (Distilled water was added instead of DNA). PCR products were analyzed by electrophoresis for 60 minutes at 90 volts on 1.5% agarose gel after staining with ethidium bromide. After performing the PCR, according to the expected size for the general (MY09/MY1) and specific (*HPV*18) primers, which is around 450 and 335 bp, the amplification resulting from the PCR with the mentioned primers confirms a 450 bp fragment for *HPV* and a 335 bp fragment for *HPV*18.

Statistical Analysis

Data were analyzed using SPSS software version 22 (SPSS Inc., Chicago, IL., USA) and the Chi-square test. The P-values less than 0.05 were considered statistically significant.

Table 2. Primers used in the present study

Gene	Gene size (bp)	Primer sequence'5 - '3	Reference
***HPV18*F**	335	5-GCGCTTTGAGGATCCAACAC-3	(28)
***HPV18*R**		5-ATTCAACGGTTTCTGGCAC-3	
MY09/MY1F	450	5-GTCCACAAGAGGGATACTGATC-3	(29)
MY09/MY1R		5-GCACCAGGGATCATAACTAATGG-3	

3. Results

In this study, out of 50 cancer tissue samples, the IHC test showed 8 (16%) samples containing E6 protein in the form of brown deposits, which were observed by light microscopy and could indicate the presence of *HPV* (Figure 1). This viral protein was not observed in any non-malignant and control samples. PCR test was performed to confirm the presence of *HPV* in the samples using general and specific primers. The presence of *HPV* was reported using general primers in 8 samples (16%) confirmed by IHC (Figure 2). However, no viral genomes were observed in non-malignant and control samples. There was a significant relationship between *HPV* infection and GC (P=0.03). Specific primers confirmed the presence of high-risk *HPV*18 in GC specimens (Figure 3), similar to IHC and PCR results with general primers. There was a significant relationship between *HPV*-PCR and *HPV*-IHC (P<0.05). *HPV*-positive samples had a mean age of 62.87 ± 9.67. Men (5 samples) had the most *HPV*-positive samples compared to women (3 samples). All *HPV*-positive samples had a histo-pathological grade of 3. Also, pathological staging (Tumor (T)) in *HPV*-positive samples was equal to T1 = 3, T2 = 6, T3 = 27, and T4 = 14. A significant relationship between *HPV* positive and a number of samples (P=0.433), sex (P=0.975), age (P=0.637), grade (P=0.130), and T (P=0.100) have not been observed.

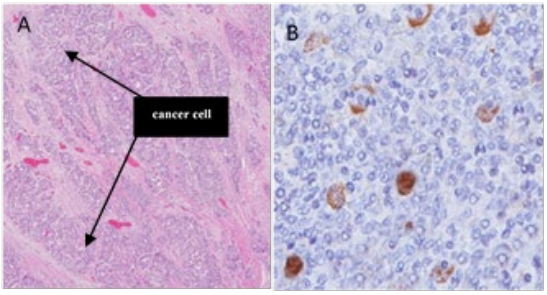

Figure 1. Identification of *HPV* 6E protein in GC samples by IHC, A: cancer cell (200x), B: cancer cell with brown deposits indicating the presence of *HPV* (400x)

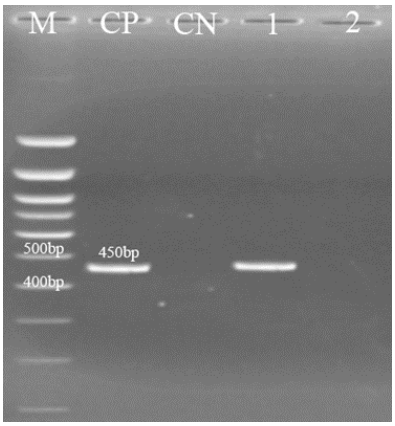

Figure 2. *HPV* agarose gel electrophoresis with general primers: M: marker size (100 bp), CP: positive control, CN: negative control, 1: positive sample, 2: negative sample

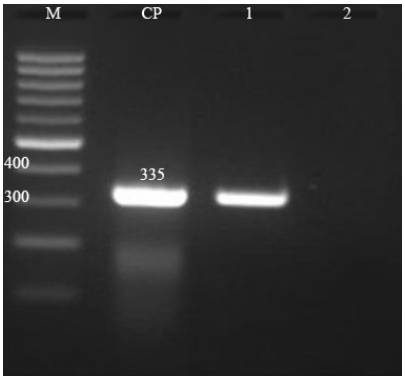

Figure 3. *HPV* agarose gel electrophoresis with specific primers: M: size marker (100 bp), CP: positive control, 1: positive sample, 2: negative sample.

4. Discussion

GC is one of the most common cancers in undeveloped regions **(26)**. In GC, factors such as diet and lifestyle primarily and bacterial and viral infections play a role in cancer risk **(30)**. Cancers associated directly or indirectly with viral infections result from changes in signaling pathways and control of cell growth. To date, no proven link between the presence of the virus and GC other than *EBV* has been clearly demonstrated. However, researchers believe

there may be a link between *HPV* infection and the development of GC similar to that found in *EBV* **(31)**. Yusefi *et al.* examined the risk factors for GC. They found that the risk factors for GC were classified into nine important categories: diet, lifestyle, genetic predisposition, family history, treatment, medical conditions, infections, demographic characteristics, Occupational exposure, and ionizing radiation **(32)**. This study investigated the presence of *HPV* in tissue samples of patients with GC and patients with benign gastric hyperplasia. The present study showed that out of 50 cancer samples, eight samples were positive for IHC and PCR tests. Sadeghian *et al*., during a study in 2022 in northwest Iran, showed that the *HPV* genome was detected in 33 (47.14%) of 70 GC samples and 4.28% (3/70) of non-GC samples, which is more than the findings of the present study. Also, in the case and control group, 97% and 67% of positive *HPV* samples were over 40 years old, and the number of men was more than women **(33)**, which is consistent with the present study. In 2018, Roesch-Dietlen *et al*. showed that of the fifty-three patients studied, *HPV* was detected in 11.32% of patients **(34)**, which is lower than the present study's findings. Leon *et al*. examined the prevalence of *HPV* in the gastroesophageal and found that *HPV* DNA was positive in one of 62 cases. The virus belonged to the *HPV16* type **(35)**. In a 2014 study, Snietura *et al*. examined the potential role of *HPV* in the pathogenesis of GC and found that out of 84 samples from gastric adenocarcinoma, *HPV* DNA replication was not observed in any of the 84 samples. IHC results also reported no expression of the p16 protein, which confirms the absence of active *HPV* infection in all individuals **(26)**, contrary to the present study's findings. Bozdayi *et al*. in 2019 **(36)**, Türkay *et al*. in 2015 **(37)**, and Erol *et al*., in 2009 **(38)**, in Turkey reported the presence of *HPV* in GC samples as 41.8%, 41%, and 9.6%, respectively, which is not consistent with the findings of the present study. In 2010, Ding *et al*. reported that 29% of 23 gastric carcinoma specimens had *HPV* DNA **(39)**, which is higher than the present study's findings. In 2021, Bae *et al*. showed that the proportion of HPV-positive cases in Chinese studies was 1.43 times higher than non-Chinese studies and 2.81 times lower than in the control group **(40)**. Examining the role of *HPV* in gastric adenocarcinoma, De Souza *et al*. showed that out of 302 samples, only 3% of the samples were *HPV*-positive, and both *HPV*16, and *HPV*18 strains were found in the samples **(41)**. In a 2003 study, Xu *et al*. found the presence of *HPV* in 68% of GC samples and even in 20% of normal gastric mucosal tissue samples **(42)**, which is higher than the findings of the present study. Yuan *et al*. **(43)** and Kamangar *et al*. **(44)** found no association between *HPV* and gastrointestinal adenocarcinoma. Therefore, the presence of *HPV* has been ruled out as GC or shown to be of little importance. Zeng *et al*., in their 2016 meta-analysis study, found that 28% of 1,917 patients with GC had *HPV* DNA. In addition, the prevalence of *HPV* in patients from China was significantly higher than in patients in non-Chinese areas **(29)**. In 2016, Fakhraei *et al*., out of 100 samples (70 men and 30 women) surveyed, five samples (four men (5.7%) and one woman (3.3%)) had *HPV* DNA **(45)**, which is lower than the findings of the present study. The diversity of data may be due to the use of different *HPV* detection methods, sample numbers, sample collection methods, different methods of protecting the sample against viral contamination, geographical differences, and various types of selected subgroups **(46, 47)**. One of the important limitations of the present study is the lack of examination of all *HPV* serotypes and the small number of samples, which limits the study's statistical power and potential bias. It is necessary to perform other studies with a high number of samples along with examining all *HPV* serotypes.

5. Conclusion

The findings of our study show the prevalence of *HPV* infection in GC in the study area. But more studies are needed to clarify the possible role of *HPV* in gastric carcinogenesis. Therefore, the positive findings of the present study may be useful to suggest further investigation of the mechanism and recommend a study of high-risk subpopulations. According to the present study's findings, it can be concluded that the IHC method and PCR have the same sensitivity for detecting *HPV*, but to replace it with the PCR method, additional studies with large sample sizes and different cancer samples are needed.

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Ethical Approval

The project was approved by the committee for ethics in biomedical research of Islamic Azad University, Kazerun Branch (IR.IAU.KAU.REC.1400.143).

Conflict of Interest

The authors declared that there is no conflict of interest regarding this article.

Authors' Contribution

AJS, ASH, HBB, BB, and BJ conceived the project and designed the experiments. AJS collected the samples. AJS performed laboratory experiments. AJS, ASH, HBB, and BB analyzed the data. AJS, ASH, and BJ wrote the draft. All authors reviewed and approved the final manuscript.

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