

Original Article

Prevalence of Human Papillomavirus Infection in Gastric Cancer in Ardebil Province, Northwest of Iran

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Abstract

Background and Aims: Human papillomavirus (HPV) is considered to be the important viral agent associated with several human cancers, the most important of which is cervical cancer. Today, the role of this virus in gastrointestinal cancers, including gastric cancer (GC), has also been considered. This study performed to clarify the possible association of HPV and the occurrence of GC in Ardabil province, Northwest Iran, which is a high-risk area.

Materials and Methods: The study involved 140 paraffin-embedded specimens of gastric tissue that divided into two groups based on the pathological diagnosis: 70 patients with GC as the case group and 70 samples without a diagnosed tumor in gastric tissue as control. The nested polymerase chain reaction (Nested-PCR) method was carried out to detect the HPV genome in paraffin embedded gastric tissue samples. Finally, samples that were positive for the presence of the HPV genome were sequenced to determine the type of virus.

Results: HPV genome was detected in 33 (47.14%) of 70 gastric cancer samples and 4.28% (3/70) of samples without gastric cancer. In case and control groups 97% and 67% of HPV positive samples were over 40 years old, respectively and the number of men was more than women. Ultimately, HPV-16 and HPV-18 were detected in PCR positive samples by sequence analysis.

Conclusion: Based on our founding, the infection rate of HPV in patients with gastric cancer was significantly higher than that in non-cancerous samples of gastric tissue. Moreover, high-risk types of HPV (16, 18) were detected in all positive samples. Therefore, the results of this investigation suggest that HPV can be one of the possible risk factors for the occurrence of gastric cancer in Ardabil province.

Keywords: Human papillomavirus (HPV), Gastric Cancer (GC), Ardabil, Iran

Introduction

Gastric cancer, due to its poor prognosis, is often diagnosed in advanced stages, and on the other hand, treatment options are limited, so the mortality rate in this cancer is relatively high. Gastric cancer is the third leading cause of cancer deaths and the

fifth most common cancer worldwide (1, 2). The prevalence of this cancer varies in different geographical areas. The disease is more prevalent in China, Japan, and other East Asian countries than elsewhere (3). Iran is also one of the high-risk areas for gastric cancer. The northern and northwestern regions of Iran are more at risk for gastric cancer. Ardabil province in the northwest of Iran has the highest rate of gastric cancer with age-standardized incidence rates (ASR) of 22.79

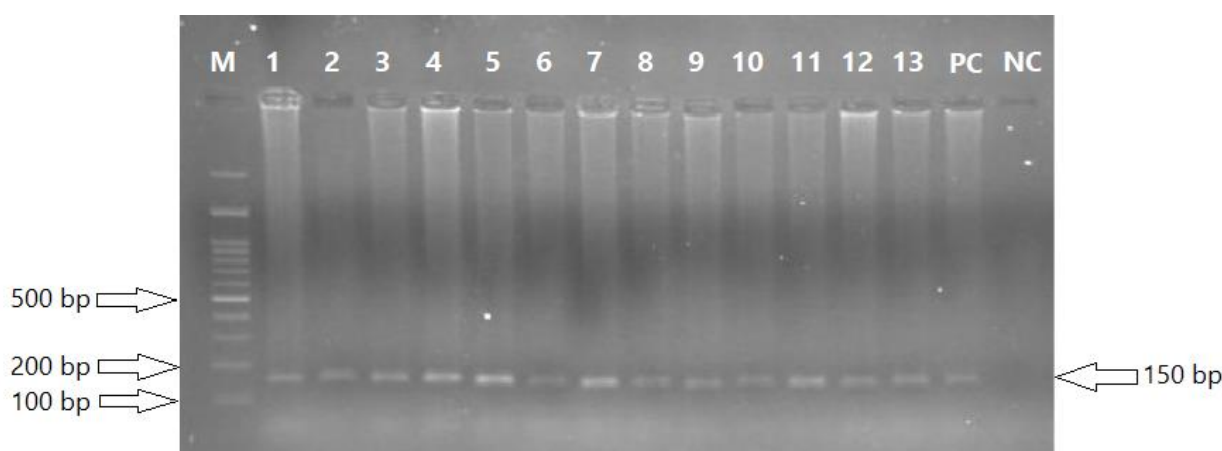
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Table 1. Primer sequences that used for the PCR amplification and their annealing temperatures and PCR product sizes.

Target region	Primer Sequences 5'→3'	Annealing temperature (°C)	PCR product size (bp)	Reference
β-Globin gene	PCO4 : 5'- CAACTTCATCCACGTTCCACC -3'	55	268	(16, 17)
	GH20 : 5'- GAAGAGCCAAGGACAGGTAC -3'			
HPV L1 gene	My09 : 5'-CGTCCMARRGGAWACTGATC-3'	55	450	(18, 19)
	My11 : 5'-GCMCAGGGWCATAAYAATGG-3'			
	Gp5+ : 5'-TTTGTTACTGTGGTAGATACYAC-3'	40	150	
	GP6+ : 5'-GAAAAATAAACTGTAAATCATATTC-3'			

bp, base pair

**Fig. 1.** Representative 1.5% agarose gel electrophoresis of Nested-PCR product for HPV detection.

Lane M, DNA ladder (100 bp); lane NC, negative control (Distilled water); lane PC, positive control (extracted DNA from HeLa cell line); lanes 1-13, positive clinical samples (150bp).

and 14.24 per100000 among men and women, respectively (4-6).

Studies show that many factors contribute to gastric cancer, including smoking, high salt intake, obesity and overweight, gastric reflux, genetic predisposition, and environmental factors such as infectious agents (*H.pylori*, EBV, HPV) (7-10). HPV is an important infectious agent in humans. This virus tends to grow in epithelial cells and is divided into 5 genotypic groups (alpha, beta, gamma, mu, and nu) based on gene sequence analysis (11, 12). Alpha is the largest genotype group that infects both cutaneous and mucosal epithelium. This group is divided into low-risk and high-risk types based on their ability to cause cancer. High-risk types are mainly classified as carcinogenic. HPV-16 and HPV-18 are the most important high-risk types of HPV(13, 14).

So far, traces of this virus have been observed in several human cancers, including cervical cancer, where almost all cases of this disease (99/97%) are caused by infection with HPV. The presence and role of this virus in gastrointestinal cancers such as gastric cancer is also under consideration (11, 15).

Methods

Study population: A total number of 140 paraffin-embedded gastric tissue samples, including 70 cancers and 70 non-cancerous specimens were collected from the pathology department of Imam Khomeini Hospital in Ardabil and were entered into our study. All specimens were diagnosed by a specialist pathologist, then transferred to Tabriz University of Medical Sciences for research.

Table 2. The description of gender, age, HPV positivity and P.Value in case and control samples

	Cancerous samples (Case) <i>Total</i> N = 70	Non cancerous samples (Control) <i>Total</i> N = 70	P.value
<u>Gender</u>			
Female	22 (31.42%)	27 (38.57%)	0.376
Male	48 (68.57%)	43 (61.42%)	
<u>Age</u>			
≤ 50	5 (7.14%)	16 (22.85%)	0.009
>50	65 (92.85%)	54 (77.14%)	
Mean age	69.88±12.46	61.05±14.99	
<u>HPV</u>			
+	33 (47.14)	3 (4.28)	<0.0001
-	37 (52.85)	67 (95.71)	
HPV, Human papillomavirus;			

Required medical information about each of the patients was obtained from the archived medical records in the pathology department. The mean age of gastric cancer patients and the control group was 69.88±12.46 and 61.05±14.99 years, respectively.

Sample Preparation:

Paraffin-embedded gastric tissue blocks were cut with thin thickness. New blades and separate disposable gloves were used to prevent cross-contamination.

Moreover, we worked under the biological hood. After cutting, the samples were collected in a sterile microtube. Xylene was used to remove paraffin then samples were washed with ethanol in two steps to remove Xylene. Finally, after complete evaporation of ethanol, the samples were prepared for DNA extraction.

DNA extraction & Quality Control: DNA extraction from paraffin-embedded tissue was performed according to the protocol of the

Yekta-Tajhiz kit (DNA Extraction from Paraffin Tissue kit, Taiwan). To prove the presence of sufficient DNA in the extracted samples and the absence of PCR inhibitors, at first, the OD of samples was read by spectrophotometer, and then PCR was carried out for the beta-Globin gene. The PCR product size was 268 bp and the nucleotide sequences of the primers are given in the table 1.

Nested-Polymerase Chain Reaction assay (Nested-PCR): After assuring the quality of the extracted DNA, we used a Nested-PCR assay to identify the HPV genome. In the first step of Nested-PCR, MY09/MY11 primer pairs were used. These primers targeted the L1 region of the HPV genome and the resulting product was 450 bp. The primer nucleotide sequences are shown in Table 1.

The amplification mixture consisted of 10x PCR buffer, Mgcl2 (25mM), dNTPs (10 mM each), Taq DNA polymerase (5 u/μl), Primer

MY09 (10 μ M), Primer MY11 (10 μ M), and 500 ng of DNA Template in a final volume of 50 μ l.

The initial denaturation step was for 5 minutes at 94°C, thereafter Thirty-eight amplification cycles were performed as follows: one minute at 94°C, one minute at 55°C, and 1 minute at 72°C. In addition, a final extension was also performed for 7 min at 72°C.

The GP5+/GP6+ primer pairs were applied in the second step of Nested-PCR, which amplify an L1 fragment of approximately 150-bp. The nucleotide sequence of the primers is given in the table1. The amplification mixture consisted of 10x PCR buffer, Mgcl2 (25mM), dNTPs (10 mM each), Taq DNA polymerase (5 u/ μ l), Primer GP5+ (10 μ M), Primer GP6+ (10 μ M), and 500 ng of DNA Template in a final volume of 50 μ l.

The initial denaturation step was for 5 minutes at 94°C, thereafter Thirty-eight amplification cycles were performed as follows: one minute at 94°C, 1.5 minute at 40°C, and 1.5 minute at 72°C. In addition, a final extension was also performed for 7 min at 72°C.

In both PCR reactions, extracted DNA from the HeLa cell line was used as HPV positive control, and Distilled water was used for negative control.

Gel electrophoresis: The PCR products were assessed by 1.5% agarose gel electrophoresis. Then agarose gels were observed under UV light by the Gel documentation system. Samples with a band of 150 bp in the gel were considered positive samples. After that, sequencing of the positive PCR products was performed to determine the type of HPV.

HPV typing and phylogeny: Direct sequencing of L1 genes was carried out (Perkin ElmerABI-3130XL DNA Sequencer, Foster City, CA, USA) using 0.5 μ L of appropriate primers GP5+ and GP6+ for a surface gene. The electropherograms were examined visually using Chromas software. Sequences of the L1 genes were aligned using BioEdit software (version 7.0.9). Genotyping was carried out by phylogenetic analysis with reference sequences of HPV genotypes.

Phylogenetic tree generation was conducted using MEGA software (version X), with the

maximum likelihood (ML) method in the Kimura two-parameter substitution matrix B (20). Bootstrap resampling and reconstruction were carried out 1000 times to confirm the reliability of the phylogenetic tree (21).

Significance was based on bootstrap values of 70. The L1 gene sequence of the bovine papillomavirus (BPV) was used as an out-group.

Statistical analysis: Statistical analyses were performed using SPSS software (Version 25). Descriptive statistics tools including percentage and frequency were used to display qualitative data and standard deviation and mean were used to explain quantitative data.

Moreover, to compare the mean age between case and control groups we used an independent t-test. In addition, Simple and multivariate logistic regression tests were performed to evaluate the relationship between HPV and gastric cancer, and match the results in terms of age, respectively.

Results

In this study, 140 paraffin-embedded gastric tissue blocks were examined. The quality of DNA extraction was admissible in all collected samples. Based on the pathologist's diagnosis, the study subjects were divided into case and control groups, which included cancerous and non-cancerous samples of gastric tissue, respectively. Of the 70 paraffin-embedded tissue specimens of gastric cancer, 33 (47/14%) were positive for the presence of the HPV DNA. In addition, the positive rate of HPV genome in the control group was three (4/28%) (Fig 1).

Clinical findings of patients are summarized in Table 2. DNA sequence analysis was performed for the Positive PCR products and sequencing results were analyzed to recognize the HPV type. In positive samples of the case group, HPV-16 and HPV-18 were detected in 22 cases (66/66%) and 11 cases (33/33%), respectively.

Also In the control group, HPV-16 and 18 were identified in two cases (66/66%) and one case (33/33%) of three positive samples, respectively. The clinical data of HPV-positive samples are detailed in Table 3. After analyzing the results obtained from the L1

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Sequencing, a Phylogenetic tree was constructed using MEGA software (version X), with the maximum likelihood method in the Kimura two-parameter substitution matrix B (Fig 2).

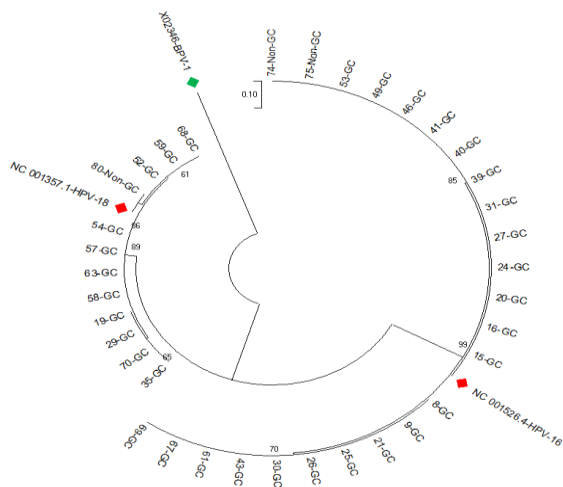


Fig. 2. Phylogenetic tree based on L1 Sequencing. Maximum likelihood phylogenetic analysis based on the alignment of the L1 amplicon of 33 and 3 HPV isolates from gastric cancer and without gastric cancer samples, respectively. Bootstrap values indicate 1000-fold replicates. Bovine papillomavirus was used as the out-group sequence.

Table 3. Correlation between the presence of HPV subtype and the Clinical and Pathologic characteristics in HPV-positive samples

HPV positive samples	Age average (Year)	Gender	HPV-16 (+)	HPV-18 (+)
Gastric cancer (33 cases)	70.8	M 21 (63.63%)	15 (71.42%)	6 (28.57%)
		F 12 (36.36)	7 (58.33)	5 (41.66)
Control group (3 cases)	60	M 2 (66.6)	2 (100%)	0 (0.00%)
		F 1 (33.33)	0 (0.00%)	1 (100%)

HPV, Human papillomavirus; F, female; M, male

Discussion

After cardiovascular disease, cancers are the second leading cause of death in the world,

among which gastric cancer is the third cause of death from cancer and the fifth most common cancer worldwide (3, 22).

Gastric cancer is one of the most important gastrointestinal cancers due to its poor prognosis, rapid progression, and limited treatment options and is a major health challenge in many countries. (1, 2).

According to global estimates, More than one million new cases of GC are diagnosed annually in the world. The prevalence of this cancer in men is approximately twice that of women (10). The highest prevalence of GC is found in East Asian countries as well as parts of Central and South America and Central and Eastern Europe (23). More than half of all GC cases occur in East Asian countries, especially China. GC has been a leading cause of death in China since 2010 because more than 80% of GC patients in China are diagnosed at an advanced stage (24, 25). In Iran, as a high-risk area, the northern and northwestern regions are more at risk of gastric cancer (26). Studies show that several factors can contribute to cancer, including infectious agents. Oncogenic viruses are one of the infectious agents that can play a role in causing cancer. Viral infections are involved in approximately 20% of all human cancers (27-29).

Gastric cancer is also a multifactorial disease, which is probably why its prevalence varies in different geographical areas. One of the possible viral agents in the incidence of gastric cancer is HPV (7, 30). Numerous studies have been performed on the association of this virus with gastric cancer.

In the studies in China and Japan that both countries are the high-risk areas for gastric cancer, the prevalence of HPV was 67.6% and 45%, respectively. In the study conducted in Japan, HPV-16, HPV-18, and HPV-33 were detected in seven cases (30%), 6 cases (26%), and 17 cases (74%) of positive samples, respectively. More than one type of HPV was detected in six cases of positive samples (31, 32).

In another study in China, 41.3% of patients with gastric cancer were positive for HPV DNA (33). These results indicated the possible role of this virus in gastric carcinogenesis.

In the present study, Cancerous and non-cancerous samples of gastric tissue were collected from Ardabil province, which ranks first in terms of cancer incidence in Iran. The results of statistical analysis (chi-square test) showed that the ratio of male to female in the case and control groups was not statistically significant ($P = 0.376$). However, the ratio of age groups > 50 years and ≤ 50 years in these two groups was statistically significant ($P = 0.009$) 77% of the control group and 93% of the case group were over 50 years old.

Examination of the samples also showed that the frequency of HPV in patients with gastric cancer was 47.14% (33/70), which was consistent with the findings of previous studies in high-risk areas of the world.

We also identified the HPV genome in 4.28% (3/70) of the control group, which included non-cancerous samples of gastric tissue.

However, the prevalence of HPV in the case group was significantly higher than the control group and the ratio of HPV in the case and control groups were statistically significantly different ($P < 0.0001$). In fact, based on these results, we can say that there is a significant relationship between gastric cancer and HPV.

In contrast, in a study in Brazil, the HPV genome was detected in only 10% of gastric cancer specimens (34). In another study in Sudan, paraffin-embedded tissue specimens of gastric cancer were investigated by Immunohistochemistry (IHC) assay. The prevalence of HPV in this study was 6.7% (35). In a study in Mazandaran, Northern Iran, 100 cases of paraffin-embedded specimens of gastric cancer tissue were examined by PCR test. Of these, five cases (5%) were positive for the HPV genome. In addition, the positive cases encompassed HPV-16 (60%), HPV-18 (20%) and HPV-45 (20%) (1).

The difference in the prevalence of HPV in the studies conducted in various geographical areas can be due to differences in research design, sensitivity and specificity of the methods used, the involvement of environmental factors, and statistical analysis.

In general, viruses can cause approximately 20% of human cancers that occur annually. HPV is considered to be one of the viral

infections associated with cancer (15, 36). So far, more than 200 different types of HPV have been identified, which are divided into high-risk and low-risk types based on their ability to cause cancer (37, 38). Low-risk types are the main cause of benign lesions, while high-risk types can play a role in carcinogenesis by interfering with cell proliferation regulatory pathways (39, 40). More than 5% of human cancers are caused by high-risk HPVs (HPV-16 and 18 are among the most important high-risk types) (41). However, the presence of some low-risk types of HPV, including types 6 and 11, has also been reported in some cancers (42).

In the present investigation, sequence analysis revealed that HPV-16 and HPV-18 were detected in 66.66% and 33.33% of HPV positive gastric cancer samples, respectively.

Our results were similar to the findings of previous studies in which the most common HPV types in gastric cancer were types 16 and 18. In addition, three cases of non-cancerous gastric tissue samples were positive for the HPV genome and were subjected to DNA sequence analysis, HPV types 16 (66.66%) (2/3), and types 18 (33.33%) (1/3) were detected.

Despite differing reports from various studies around the world, the results of most studies suggest a possible role for HPV in gastric cancer. Although in some studies, a strong and significant relationship was not observed between the presence of HPV and GC and the prevalence of this virus in GC was low, but in the same small amounts, HPV type 16 and/or 18 were identified which are among the most important high-risk types of HPV (1, 34). As a result, even in these studies, the role of HPV in gastric cancer cannot be completely ruled out.

The results of the present study also showed that the prevalence of HPV in gastric cancer samples was approximately 11 times higher than non-cancerous gastric tissue samples. Therefore, a significant difference was observed in the prevalence of HPV in the case group compared to the control group.

After sequencing of positive samples, the most important high-risk types of HPV (types 16 and 18) were identified. Therefore, based on

the evidence obtained, it can be concluded that HPV can probably be one of the effective factors in the incidence of gastric cancer in Ardabil province.

Conclusion

According to our study, there was a close association between gastric cancer specimens and high-risk HPV types 16 and 18, which could be a warning to people in the control group who were positive for high-risk HPV types.

These individuals should be continuously monitored and followed up so that if abnormal cellular changes are observed, effective measures can be taken as soon as possible to prevent and treat them. It is also suggested that more extensive studies be performed in other parts of the world to more accurately determine the role of HPV in gastric carcinogenesis.

In addition, to prevent HPV-related cancers, vaccination against this virus should be pursued more seriously.

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Conflict of interest

The authors declare they have no conflict of interest.

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