RESEARCH ARTICLE

Presence of Human Papillomavirus DNA in Colorectal Cancer Tissues in Shiraz, Southwest Iran

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Abstract

Background: Colorectal cancer is one of the most common cancers worldwide. Viruses including human papillomavirus (HPV) have been reported to be associated with different cancers but any association with colorectal cancers remains controversial. Aim: To evaluate any association between HPV infection and adenocarcinoma of the colon and adenomatous polyps. Materials and Methods: Paraffin-embedded tissue specimens of 70 colorectal adenocarcinomas, 70 colorectal adenomatous polyps, and 70 colorectal normal tissues were subjected to DNA extraction. The quality of the extracted DNA was confirmed by amplification of a β-globin fragment using polymerase chain reaction (PCR). PCR using specific primers were performed to detect HPV DNA. Specific primers targeting the E6 region of the HPVs 16 and 18 were used for genotyping. Results: HPV DNA was detected in 2 (2.85%) out of 70 adenocarcinoma colorectal tissues and 4 (5.71 %) out of 70 adenomatous colorectal tissues. All normal colorectal tissues were negative for HPV DNA. HPV-16 was the most predominant genotype (5 sample) followed by HPV-18 (4 sample). Despite the above observations, statistical analyses indicated no significant differences in the frequencies of HPV positive subjects between the cancerous and normal samples. Conclusions: Although the differences observed in the frequencies of HPV positive cases in our study was not significant relative to those of control subjects, the fact of 6 positive samples among cancerous tissues, may still suggest a role of HPV in colorectal carcinogenesis. The study collectively indicated that some colorectal cancerous tissues are infected with high risk HPV genotype. The findings merit more investigation.

Keywords: Colorectal cancer - HPV - adenocarcinoma - adenomatous polyp

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Introduction

Cancer is a term that includes a number of clinically and biologically different conditions in which the fundamental mechanisms of growth control are largely disrupted while the growth ability is somewhat retained. In 2008, more than 10 million people were expected to be diagnosed with cancer worldwide. 90-95% of cancers were caused by the environment and lifestyle factors and 5-10% had their roots in genetic alterations (Preetha Anand, 2008).

Colorectal cancer (CRC) is one of the most common malignancies and major cause of morbidity and mortality throughout the world. Worldwide, CRC is represented 9.4% and 10.1% in men and women, respectively (Boyle and Langman, 2000). In 2008, CRC was ranked as the third cancer in men and the second cancer in women, with over 1.23 million new cases per year (Center et al., 2009). There are many risk factors for development of colorectal cancer such as advanced age, country of birth, long-standing ulcerative colitis, high red meat diet, obesity, and cholecystectomy (Haggar and Boushey, 2009; Jemal et al., 2011).

It has been estimated that 20% of all cancers are caused by infectious agents including viruses. Viruses, especially DNA viruses such as polyomaviruses, papillomaviruses (HPV), Epstein-Barr virus and hepatitis B virus, are the causative agents of 10%-15% of human cancers including Merkel cell carcinoma, cervical cancer, Burkitt's lymphoma, Hodgkin's lymphoma, nasopharyngeal carcinoma, and hepatoclellular carcinoma (Antonic et al., 2013; Chen et al., 2014; Sarvari et al., 2014).

Genomic instability including accumulation of mutations, aberrations and DNA damage are the most common outcome for virus-induced reprogramming in the infected cells. Although each virus has a specific mechanism for cancer induction, most of DNA oncogenic viruses contain oncogenes which promote transformation of the infected cells, mostly by disruption of protein function such as p53 and pRb (Chen et al., 2014). Recently, a number of studies have shown that some viruses such as HPV, BK, JC and EBV may be related to the outcome of colorectal cancer (Antonic et al., 2013).

HPV as an infectious agent may infect the basal layer of the epithelial cells and play a role in carcinogenesis (Stanley, 2012). There are several different types of

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papillomaviruses, some of which are associated with cancer including papillomavirus types 16, 18, 31, 33, and 35 (Antonic et al., 2013). The mechanism of carcinogenicity of papillomaviruses depends on the viral integration into the DNA of the host cells and expression of the oncoproteins E6 and E7, which disrupt the functions of the tumor suppressor proteins p53 and pRb, respectively (Munger et al., 2004). Investigations have indicated that HPVs has an important role in benign and progression of malignant lesions of the urogenital tract and the head and neck region (Egawa et al., 2015). Moreover, studies have shown that HPV might be related to development of other malignancies, such as breast, esophageal and bladder cancers (Heng et al., 2009; Barghi et al., 2012; Guo et al., 2012). The results of some studies suggest that HPV DNA is present in colorectal neoplasia (Kirgan et al., 1990; McGregor et al., 1993; Cheng et al., 1995; Damin et al., 2007; Meshkat et al., 2014; Ranjbar et al., 2014); other studies, however, were not able to detect HPV DNA in colorectal cancers tissue (Andrea N. Burnett-Hartman, 2011; Taherian et al., 2014).

Cancer is the third cause of death in Iran. Following lung and breast cancer, colorectal cancer is the third most common cause of cancer in Iran (Mousavi et al., 2009). The incidence rate of colorectal cancer in Iran is 5000 new cases per year and mortality rate is reported to be 2 per 100,000 (Esna-Ashari F, 2009). Although there are some reports from Iran regarding HPV and colorectal cancer but the results are controversial. This study was conducted to evaluate the association between HPV infection and adenocarcinoma/adenomatous colorectal cancer in a population from the southwest of Iran.

Materials and Methods

From January 2012 to December 2013, 210 paraffin-embedded biopsy specimens including adenocarcinoma colorectal tissue (n=70), adenomatous colorectal tissue (n=70), and normal colorectal tissue (n=70) were collected and included in this study. Samples were collected from Faghihi hospitals, a teaching hospital affiliated with Shiraz University of Medical Sciences, according to the pathology report (Figure 1).

The study was approved by the Ethics Committee of the University and informed consent was obtained before sample collection.

DNA extraction

Seven sections (10 μm) of the paraffin-embedded block were cut and placed in a 1500 μl Eppendorf tube. The first step of deparaffinization was performed by adding 1200 μl of xylene to the 1.5-ml tubes containing the tissue section. After tube vortex and incubation for 5 min at room temperature, the tubes underwent centrifugation at 14000 rpm for 5 min. Then, the supernatant was removed and 1000 μl of 100% ethanol was added to each tube and incubated for 5 minutes at room temperature. Finally, the tubes underwent centrifugation at 14000 rpm for 5 min and the supernatant was removed. Both steps were repeated once. In the final step, the tubes were incubated at 37°C on a heating block until the ethanol was totally evaporated. The DNA was then extracted using a QIAamp DNA minikit (Qiagen, Dusseldorf, Germany) according to the manufacturer's instructions. The extracted DNA was stored at -20°C until use.

Polymerase Chain Reaction (PCR)

All the extracted DNA samples were initially subjected to PCR with consensus primers PCO3/PCO4 (β-globin) to confirm the quality of the extracted DNA. PCR was performed in a total volume of 25μL, containing 1mM MgCl2 (CinnaGene, Iran), 200 μM (each) deoxyribonucleotide triphosphates solution (dNTPs) (CinnaGene, Iran), 1X reaction buffer (CinnaGene, Iran), 1U Taq DNA polymerase (CinnaGene, Iran) and1μM each specific primer (PCO3 and PCO4) (Table 1).

PCR tests for β-globin were carried out as follows: 10 min initial denaturation at 94°C, 35 cycles of denaturation at 94°C for 45s, annealing at 44°C for 45 s, extension at 72°C for 1min, and final extension at 72°C for 10 min. Then, amplification was done on samples which were positive for β-globin gene using HPV specific primers, MY09 and MY11, which amplify L1 region (Table 1). PCR tests for L1 region were carried out as follows: 5 min initial denaturation at 95°C, 50 cycles of denaturation at 95°C for 1min, annealing at 48°C for 1min and 45 s, extension at 72°C for 3.5 minutes , and final extension at 72°C for 8 min. For determination of HPV genotypes, we performed two sets of PCR using HPV16 and HPV18 specific primers which amplify E6 region (Table 1)as follows: 5 min initial denaturation at 94°C, 50 cycles of denaturation at 94°C for 1min, annealing at 54°C for 1min, extension at 72°C for 1.5 minutes, and final extension at 72°C for 5 min. PCR products were then loaded into 1.5% agarose gel and stained with 1% ethidium bromide and visualized under UV light (Figure 2).

Statistical analysis

Data were analyzed using SPSS 16 (SPSS Inc., Chicago, IL, USA) software. Fisher's exact test was used for data analysis. A P-value below 0.05 was considered statistically significant.

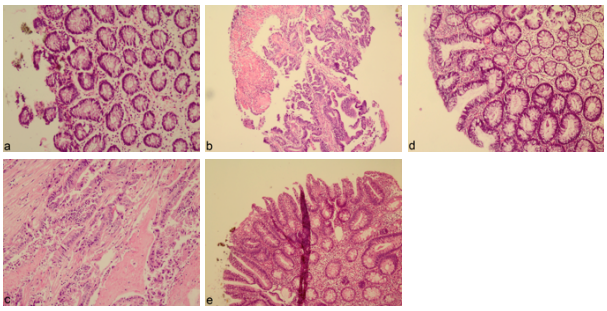

Figure 1. Photomicrograph of human colorectal tissue. A) A photomicrograph of human normal colorectal mucosa which is negative for the presence of HPV DNA. **B)** A photomicrograph of human colorectal adenocarcinoma demonstrating the presence of HPV DNA. **C)** A photomicrograph of human colorectal adenocarcinoma negative for HPV DNA. **D)** A photomicrograph of human colorectal adenomatous demonstrating the presence of HPV DNA e: A photomicrograph of human colorectal adenomatous negative for HPV DNA

Table 1. The Sequences and other Characteristics of Primers Used in this Study

Locus	Primers	5' to 3' Sequence	Size, bp
β-globin	PCO3	5'- ACACAACTGTGTTCACTAGC-3'	110
	PCO4	5'- CAACTTCATCCACGTTCACC-3'	
HPV	MY09	5'- CGTCCMARRGGAWACTGATC-3'	450
	MY11	5'-GCMCAGGGWCATAAYAATGG-3'	
Genotype 16	16F	5'- TCAAAAGCCACTGTGTCCTG-3'	120
	16R	5'- CGTGTTCTTGATGATCTGCA-3'	
Genotype 18	18F	5'- GACACATTGGAAAAACTAAC-3'	140
	18R	5'- TAGTGCCCAGCTATGTTGTG-3'	

Table 2. Frequency of Human Papilloma Virus in Samples from Different Anatomic Sites

Anatomic sites	Samples size	HPV positive
Colon	89	3
Rectum	28	2
Sigmoid	24	1
Other	69	0

Table 3. Frequency of HPV Genome among Different Groups

Tissue type	positive for HPV	Mean age
Adenocarcinoma (N=70)	2/70	56
Adenomatous (N=70)	4/70	57
Normal (N=70)	0/70	42

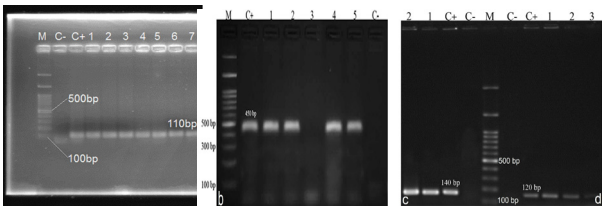

Figure 2. Photographs of gel electrophoresis. A) PCR analysis of DNA samples extracted from colorectal tissue using β- globin primers. **B)** PCR analysis of DNA samples extracted from colorectal tissue using HPV common primers. **C)** PCR analysis of DNA samples extracted from colorectal tissue using type-specific primers for HPV-18. **D)** PCR analysis of DNA samples extracted from colorectal tissue using type-specific primers for HPV-16

Results

Of 210 subjects who were enrolled in this study, 112 were male and the rest were female. Patients' ages ranged between 22 and 87 years and the mean age was 52 ± 1.64 SD years. Of 140 subjects of the study group, 77 cases were male and 63 were female; of 70 individuals of control group, 35 were male and the rest were female. The samples were removed from anatomic locations including 89 of 210 (42.38%) colon tissue, 28 of 210 (13.33%) rectum tissue, 24 of 210 (11.42%) sigmoid tissue, and 69 of 210 (32.85%) other tissues (Table 2). The extracted DNAs that were positive for ß-globin gene were subjected to HPV genome detection. The HPV genome was identified in 6 out of 210 (2.85%) samples including 2 out of 70 (2.85 %) adenocarcinoma and 4 out of 70 (5.71%) adenomatous polyps. HPV DNA was not found in any normal biopsy specimens (Table 3), using the MY09/MY11 primer set.

Statistical analysis did not show any significant differences in the frequency of HPV DNA between adenocarcinoma colorectal tissue and adenomatous colorectal tissue (P=0.68). Analysis also revealed no differences in the frequency of HPV DNA between adenocarcinoma colorectal tissue and normal colorectal tissue (P=0.49). Also, when we compared the frequency of HPV DNA in adenomatous colorectal tissue and normal colorectal tissue, we could not find a statistically significant difference between the two groups (P=0.12). Totally, although HPV DNA was detected in only cancerous tissues (adenomatous or adenocarcinoma) and the frequency of HPV DNA was higher in theses tissues than normal tissues (6 versus 0), this difference was not statistically significant (P=0.18). All positive cases had high risk HPV genotypes (16 and 18) identified by PCR with E6 region specific primers. HPV-16 was the most predominant genotype, being present in 5 out of 6 positive cases (83.33 %). HPV-18 was detected in 4 positive samples (66.66 %). Four samples were co-infected with both genotypes. Although the frequency of HPV DNA was higher in female patients (4 of 63, 6.34%) than male's patients (2 of 77, 2.59%), the difference was not statistically significant (p=0.41). The mean age of the HPV positive patients was 58 years and that of all subjects was 52 years. The anatomic locations of positive samples were: 3 (50%) from the colon tissue, 2 (33.33 %) from rectum and 1 (16.66 %) from sigmoid tissue.

Discussion

Cancer is the important cause of death in both economically developed and developing countries (Jemal et al., 2011). Worldwide, colorectal cancer is one of the most common cancers with more than 1 million new cases yearly and mortality rate of nearly 33% in the developed world (Parkin et al., 2005). In Iran, colorectal cancer is one of the five most common cancers, ranking third after lung cancer and breast (Kolahdoozan et al., 2010).

HPV is a DNA virus and has been suggested to be associated with cancers such as cervical cancer, anal cancer, and mouth and throat cancer (Munoz et al., 2006). Several studies from different parts of the world such as the United Stated, Brazil, China and Argentina have detected HPV genome in tissue samples of colorectal cancer and concluded that HPV might be involved in disease progression (McGregor et al., 1993; Cheng et al.,

1995; Damin et al., 2007; Ghasemian et al., 2013). In the present study, we determined the frequency and genotypes of HPV virus in tissue samples of adenocarcinoma, adenomatous and normal colorectal tissues.

In our study, HPV DNA was detected in 2 out of 70 (2.85 %) adenocarcinoma colorectal tissues and 4 out of 70 (5.71%) adenomatous colorectal tissues. Statistical analysis did not show significant differences in the frequency of HPV DNA between adenocarcinoma colorectal tissues and adenomatous colorectal tissues. In a study performed by Kirgan, 29 out of 30 (97%) adenocarcinoma colorectal tissue and 18 of 30 (60%) adenomatous colorectal tissue were positive for HPV (Kirgan et al., 1990). Also, in the study performed by Cheng et al., 37 out of 70 (53%) adenocarcinoma colorectal tissues and 11 out of 37 (29%) adenomatous colorectal tissues were positive for HPV (Cheng et al., 1995). In a study performed by McGregor et al. 13 out of 38 (32%) adenocarcinoma colorectal tissues and 8 out of 21 (38%) adenomatous colorectal tissues were positive for the presence of HPV (McGregor et al., 1993).

In our study, all the normal colorectal tissues were negative for HPV DNA. The frequency of HPV DNA positive cases between adenocarcinoma colorectal tissues and normal colorectal tissues was not significant. In a study performed by Ranjbar et al., 5 out of 80 (6.25%) adenocarcinoma colorectal tissues and 1 out of 80 (1.25%) normal colorectal tissues were positive for the presence of HPV (Ranjbar et al., 2014). This finding is consistent with our finding. Also, in a study performed by Kirgan et al., 29 out of 30 (97%) adenocarcinoma colorectal tissue and 7 out of 30 (23%) normal colorectal tissues were positive for the presence of HPV (Kirgan et al., 1990). McGregor et al. reported the presence of HPV DNA in 13 out of 38(32%) adenocarcinoma colorectal tissues and 2 of 24(8%) normal colorectal tissues (McGregor et al., 1993).

When we compared the frequency of HPV DNA positive cases in adenomatous colorectal tissues and normal colorectal tissues, we could not find a statistically significant difference between the two groups. In another study performed by McGregor et al., 8 out of 21(38%) adenomatous colorectal tissues and 2 out of 24 (8%) normal colorectal tissues were positive for the presence of HPV (McGregor et al., 1993). In the study by Kirgan et al., 18 out of 30 (60%) adenomatous colorectal tissues and 7 out of 30(23%) normal colorectal tissues were reported to be positive for the presence of HPV in the United States (Kirgan et al., 1990).

Adding together the colorectal cancer tissues, adenomatous or adenocarcinoma, although the frequency of HPV DNA was higher in comparison to normal tissues (6 versus 0), this difference was not statistically significantly in our study.

Meshkat et al. showed that HPV DNA was present in 1 out of 100 (1%) of colorectal cancer tissues (Meshkat et al., 2014). Results of the study performed by Ranjbar et al. revealed that HPV DNA was present in 5 out of 80 (6.25%) cancerous colorectal samples and 1 out of 80 (1.25%) control subjects (Ranjbar et al., 2014). Taherian et al. showed that HPV DNA was not present in any of the normal, adenocarcinoma, or adenoma samples in the population from Tehran, Iran (Taherian et al., 2014). In the study reported by Andrea et al. (2011). HPV DNA was not found in any of the 609 colorectal tissue samples and control samples (Andrea N. Burnett-Hartman, 2011). In other studies conducted by McGregor et al., Kirgan et al., Damin et al., 32%, 97%, 83% of colorectal cancer tissues and 8%, 23% and 19% of healthy control groups were positive for HPV DNA and HPV antigen, respectively. These studies have demonstrated a significant association between the presence of HPV and colorectal cancer (Kirgan et al., 1990; McGregor et al., 1993; Damin et al., 2007).

Several studies reported the association between HPV and colorectal cancer, and some studies did not support this subject. This controversy may come from the differences in genetic background of the patients, geographical differences, and the differences in the sample size and cultural differences (sexual behavior) of the patients in different studies. Diversity may also be explained by the possibility of contamination during sample preparation and testing as well as differences in the frequency of HPV infection among different populations with different sexual behaviors.

In our study, HPV-16 was the most predominant genotype (5 sample) associated with the disease followed by HPV-18 (4 sample). Our results indicated genotypes 16/18 as the most common genotypes isolated from tissue samples; this finding is consistent with those of Ranjbar et al., Damin et al., Cheng et al. and Young Cheng et al. (Cheng et al., 1993; Cheng et al., 1995; Damin et al., 2007; Ranjbar et al., 2014).

In our study, of the 6 positive HPV samples from adenocarcinoma/ adenomatous colorectal tissues, 3(50%), 2(33.33%) and 1(16.66%) samples were the tissues from the colon, rectum and sigmoid, respectively. In the study performed by Kirgan et al., of 54 positive HPV samples of adenocarcinoma/ adenomatous and normal colorectal tissues, 19(35.18%), 18(33.33%) and 17(31.48%) were from the rectum, sigmoid and cecum, respectively (Kirgan et al., 1990). Also, in the study performed by Damin et al., of 60 positive HPV samples of adenocarcinoma colorectal tissue, 28(46.66%) were from the colon and 32(53.33%) from the rectum (Damin et al., 2007). The study performed by Ranjbar et al. revealed that the presence of HPV in the colon tissue is higher than those from rectal tissues (80% versus 20%) (Ranjbar et al., 2014). These findings are consistent with our results indicating that the presence of HPV in the rectal tissue was higher compared with other tissues.

In the present study, out of 6 HPV positive samples, 4 and 2 were from females and males, respectively. Although the frequency of HPV positive cases was higher in colorectal cancer tissues from females than males, the difference was not statistically significant (p=0.41). In the study performed by Damin et al. out of 60 positive HPV samples, 31 and 29 samples were from males and females, respectively (p=0.75) (Damin et al., 2007).

In conclusion, although the differences observed in the frequencies of HPV positive cases in our study was not significantly different from those of the control subjects, observing 6 positive samples among cancerous tissues, but

not normal control tissues, may still suggest the role of HPV in colorectal carcinogenesis. The study collectively indicated that some colorectal cancerous tissues are infected with high risk HPV genotype, HPV 16 and 18. The findings merit more investigations.

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