RESEARCH ARTICLE

The Aetiological Role of Human Papillomavirus in Colorectal Carcinoma: An Iranian Population- Based Case Control Study

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Abstract

Background: Human papillomavirus (HPV) is one of the most common sexually transmitted infections worldwide and the association between HPV infection and genital cancers has been well established. This study concerned the possible role of HPV infection in colorectal carcinoma (CRC) in the Iranian population. Materials and Methods: We examined 80 tissues obtained from patients with colorectal cancer consisting of 58 colon cancer samples and 22 rectal cancer samples and 80 tissues from patients with unremarkable pathologic changes as matched controls by sex, study center and anatomical sites. HPV infection and genotypes were detected using nested PCR and sequencing methods, respectively. Results: HPV DNA was detected in 5/80 (6.25%) cases including 1 of 22 (4.54%) patients with rectum cancer and 4 of 58 (6.9%) patients with colon cancer and 1/80 (1.25%) of controls. Furthermore, HPV-18 was detected as the most frequent type and we found no significant correlation between prevalence of HPV infection and anatomical sub- sites. Conclusions: Although a causal relation between human papillomavirus and colorectal cancer was not found through this study, analysis of medical records pointed to a possible role for high- risk types of HPV in increasing the potential of aggressiveness in colorectal cancer. This study shows a particular frequency of HPV genotypes in patients with colorectal cancer in Iran. Since HPV vaccines are limited to a few types of virus, using cohort studies in different geographical zones to screen for patterns of HPV infection in different organs might increase the efficacy and optimization of the current vaccines.

Keywords: Human papillomavirus - colorectal cancer - Iranian population - HPV vaccines

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Introduction

Colorectal cancer is the third leading cause of cancer dependent death in the world and there are one million new cases diagnosed per year (Burnett-Hartman et al., 2008; Giuliani et al., 2008) and also colorectal cancer is the third common cancer in Iranian population (Motlagh et al., 2007). It is a complex of multistep and multifactorial events, as an interaction between environmental and life style, sequential genetic alternations and viral infection (Giuliani et al., 2008; Doosti et al., 2011). In the past decade, different scientists have investigated an association between HPV infection and colorectal cancer (Chen et al., 2012), also colorectal cancer originates in epithelial cells and anal tissues, a site known to be associated with HPV-related malignancies (Burnett-Hartman et al., 2011), but they have screened completely inconsistent results. Some of the studies reporting 14-84 % of colorectal neoplasia positive for HPV DNA, and others detecting slight or no HPV DNA presence in colorectal cancer and adenomatous polyps. Therefore, the association between HPV infection and colorectal cancer is not clear (Burnett-Hartman et al., 2011).

Papillomaviruses are a group of genetically related organisms, which infect epithelium and infuse proliferation variation in infected cells, which can lead tissues in both benign and malignant tumors (Al-Maghrabi, 2007). Currently, types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82 classified as high- risk types; types 6, 11, 40, 42, 44, 54, 61, 70, 72, 81 and CP6108 are classified as low- risk types; and types 26, 53 and 66 considered as probably oncogenic (Burnett-Hartman et al., 2008; Yahyapour et al., 2013). The oncogenic potential of HPV is related to its ability to interfere, upon viral integration into the host cell DNA, with the cell cycle and tumor supportive function of the p53 and pRB proteins (Giuliani et al., 2008). Furthermore, investigators discovered a putative p53 binding site within the promoter region of KAI1 and suggested that p53 directly activates the expression of the metastasis suppressor KAI1 (Mashimo et al., 1998).

Identification of HPV as a predisposing factor for colorectal malignancies would have significant

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implications in human health, allowing the opportunity to detection high risk groups or development of novel therapeutic modalities and create positions to better understanding the biology and mechanism of the diseases (Motlagh et al., 2007). Furthermore, finding the association between colorectal carcinoma with specific infectious agents may provide new aspects to investigate more effective cancer prophylactic strategies (Burnett-Hartman et al., 2008).

We attempted to design the first case- control study by effective sample size in Iran to cast further light on the detection of HPV role in colorectal carcinoma.

Materials and Methods

Study population

Data derived from previous investigations which were performed about the frequency of HPV DNA in colorectal cancer in different geographical zones was analyzed and conducted us to calculating the sample size. Specimens were collected from patients of Baqiyatallah Hospital of Tehran city. All patients were interviewed by inspectors using a questionnaire to achieve demographic information and they had negative histories of exposure to either chemotherapy or radiotherapy prior to surgery. Colorectal formalin- fixed and paraffin embedded tissues were obtained from 80 patients with tumors in cecum and ascending colon, transverse colon, descending and sigmoid colon and overlapping and unspecified origin as colon cancer and rectal cancer defined as tumors occurring at the rectosigmoid junction or rectum. The control group consisted of 80 patients without remarkable pathological changes were selected (1:1) at the time of diagnosis of the cases and were matched by sex, study center and anatomical sites. Colorectal adenocarcinoma and unremarkable pathological changes were confirmed by pathologists through standard criteria. The demographic data and medical information including age, gender, location, anatomical sub- sites, tumor stage, differentiation grade, lymph node and metastasis were collected from patient's medical records are shown in Table 2.

DNA extraction

5-10 slides (depends on type of sampling: surgery or needle biopsy) of about 5 μm wide were deparaffinized in xylene and absolute ethanol, then DNA was extracted using QIAamp DNA FFPE Tissue Kit (Qiagen GmbH, Germany). Several points were carefully considered to decrease the risk of sample- to- sample cross-contamination, like limiting the tissue DNA extraction process to 6 samples per day. Value index and purity of extracted DNA were studied by Nanodrop (Thermo Scientific, NanoDrop 1000, Wilmington, USA) and DNA quality was evaluated by PCR using PCO3/ PCO4 primers which can amplify a product from the human ß- Globin gene as internal controls aiming to confirm the presence of an amplifiable nucleic acid (Table 1).

HPV detection and genotyping

Detection of HPV DNA was carried out through MY09/MY11 and GP5+/GP6+ primers by Nested PCR (Table 1). The MY09/MY11 primers amplify the L1 gene of HPV, which capable of amplifying a wide spectrum of HPV types to produce a PCR product of 450 bp. The PCR reactions for MY09/MY11 primers were performed using the following steps; 5 min at 94˚C; then 40 cycles at 94˚C for 1 min, 55˚C for 1 min and 72˚C for 1 min. Finally reactions were carried out to a final extension for 5 min at 72˚C. Also nested PCR was completed by amplified GP5+/GP6+ primers. The GP5+/GP6+ primers are a non-degenerate primers set, that detect a wide range of HPV types using a lower annealing temperature, during PCR and produce a PCR product of approximately 150 bp. Second round of PCR reactions were as follows; 4 min at 94˚C; then 38 cycles at 94˚C for 1 min, 40˚C for 2 min and 72˚C for 2 min and reactions were carried out to a final extension for 4 min at 94˚C. Each batch of samples included negative controls without a DNA template; and one positive control containing HPV- 18 approved by WHO. PCR products were separated by electrophoresis through 1% and 2% Agarose gel respectively and then HPV positive samples were detected. The purified products were subjected to direct sequencing by Macrogen Inc. (Seoul, Korea) and then the HPV sequences were analyzed by the BLAST program available at the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST).

Statistical analysis

The presence of HPV DNA among cases and controls were tested by chi-square test and the association of HPV infection and HPV type with anatomical sub- sites, age, location, differentiation grade, tumor stage, distant metastasis and lymph-node metastasis were analyzed by independent samples t- test and chi-square test. All statistical analysis was performed with SPSS 16 and Microsoft Excel 2010. The significance level was set at $p<0.05$.

Results

Patients

Formalin-fixed paraffin embedded tissue samples of 80 CRC patients with mean age 62.36±6.63 years and 80 controls with mean age 52.7±6.4 years were enrolled in this study. As shown in Table 2, we found no particular differences between cases and matched controls with respect to age and location.

Genomic DNA quality control

The DNA purity and quality were confirmed for all samples.

Nested PCR analysis of HPV in colorectal samples

HPV DNA was found in 5 of 80 (6.25) cases including 1 of 22 (4.54%) patients with rectum cancer and 4 of 58 (6.9%) patients with colon cancer and also 1 of 80 (1.25%) control samples in colon tissue (1.72%). There was no significant difference between cases and controls regarding the presence of HPV DNA, $p>0.05$. The mean±SD differentiation grade and tumor stage were 1.8±0.84 and 3±1 in HPV L1 positive cancerous patients, respectively. Distant metastasis was seen in 60% of HPV

Table 1. Sequence of Primers Used

Primers	Sequences
1. ß- Golubin (F)	5'-TGG GTT TCT GAT AGG CAC TGA CT-3'
2. ß- Golubin (R)	5'-AAC AGC ATC AGG AGT GGA CAG AT-3'
3. MY09	5'-CGT CCM AAR GGA WAC TGA TC-3'
4. MY11	5'-GCM CAG GGW CAT AAY AAT GG-3'
5. GP5+	5'-TTT GTT ACT GTG GTA GAT ACT AC-3'
6. GP6+	5'-AAA AAT AAA CTG TAA ATC ATA TTC-3'

Table 2. Demographic Data and Medical Records of Patients with CRC and Controls Entry into Study

Variable	Colorectal carcinoma (n:80)		Controls (n:80)	
	HPV (+) (n:5)	HPV (-) (n:75)	HPV (+) (n:1)	HPV (-) (n:79)
Mean age (SD)	66.3±4.2	62.1±6.8	27	53.1±6.4
Gender				
Male	3 (60%)	47 (62.67%)	0	50 (63.3%)
Female	2 (40%)	28 (37.33%)	1 (100%)	29 (36.7%)
Location				
Urban	3 (60%)	46 (61.33%)	0	44 (55.7%)
Rural	2 (40%)	29 (38.67%)	1 (100%)	35 (44.3%)
Anatomical sub- sites				
Cecum and ascending	3 (60%)	10 (13.33%)	0	13 (16.45%)
Transverse	0	6 (8%)	0	7 (8.86%)
Descending	0	4 (5.33%)	0	6 (7.6%)
Sigmoid	1 (20%)	27 (36%)	1 (100%)	27 (34.18%)
Rectum	1 (20%)	21 (28%)	0	22 (27.85%)
Unspecified	0	7 (9.33%)	0	4 (5.06%)
Tumor stage				
T1	0	13 (17.33%)	-	-
T2	2 (40%)	26 (34.67%)	-	-
T3	1 (20%)	31 (41.33%)	-	-
T4	2 (40%)	5 (6.67%)	-	-
Differentiation grade				
1	3 (60%)	18 (24%)	-	-
2	2 (40%)	47 (62.67%)	-	-
3	0	10 (13.33%)	-	-
Lymph node				
Present	4 (80%)	31 (41.33%)	-	-
Absent	1 (20%)	44 (58.67%)	-	-
Distant metastasis				
Present	3 (60%)	9 (12%)	-	-
Absent	2 (40%)	66 (88%)	-	-

Table 3. HPV Positive Patients Characteristics

Variable	HPV Positive Samples					
	1	2	3	4	5	6
HPV- type	18	18	31	45	18	11
Age (year)	48	59	62	80	76	27
Gender	Male	Female	Female	Male	Male	Female
Location	Urban	Rural	Rural	Urban	Urban	Rural
Colorectal tissues	Ascending	Sigmoid	Rectum	Cecum	Ascending	Sigmoid
Tumor stage	3	4	2	2	4	-
Differentiation grade	1	2	1	2	1	-
Lymph node	Present	Present	Present	Absent	Present	-
Metastasis	Absent	Present	Present	Absent	Present	-

positive samples, p=0.027.

Sequencing analysis

Using the sequencing method, a total of three high-risk genotypes including 18, 31 and 45 and one low-risk genotype including 11 were found respectively in patients with CRC and matched controls. Evaluation of the association between anatomical sub- sites and HPV prevalence was carried out in these cases. Among colon cancer specimens with high risk HPV infection, HPV-18 was the most prevalent genotype in ascending with 2 cases and also one infection in sigmoid (Table 3).

Discussion

The main findings of this study were that HPV infection may play a role to increase the potential of aggressiveness and metastasis in colorectal cancer and also geographical variation might be related to type variation of HPV infection.

Demographic data including age, sex and location in patients with colorectal carcinoma in present study are comparable to reported by Boyle and Leon (2002), Haggar and Boushry (2009) and Almeida and Barry (2010), which indicate that more than 90 % of colorectal cancer are older than 50 years of age and the cancer incidence rate among men is slightly higher than women, hence this study has explained a similar epidemiological pattern of colorectal cancer incidence in Iranian population as several studies in this background.

In the present study, HPV DNA was detected in 6.25% of samples with colorectal carcinoma and 1.25% of matched controls and we found no significant evidences in the role of HPV infection between CRC and control group in the analyzed samples. This is in keeping with the findings of Gornick et al. (2010), Burnett-Hartman et al. (2011) and Burnett-Hartman et al. (2013), but some have investigated a significant role for HPV infection in incidence of colorectal carcinoma (Bodaghi et al., 2005; Damin et al., 2007; Motlagh et al., 2007; Chen et al., 2012). This diversity has been explained according to possibility of contamination in sample preparation and testing and different frequency of HPV infection between populations which may be affected of high- risk sexual behaviors (Bodaghi et al., 2005; Burnett-Hartman et al., 2008; Burnett-Hartman et al., 2011).

This study is the first case- control study, which investigate the role of HPV infection in colorectal cancer in Iranian population. The most frequent infecting HPV type in patients with CRC was HPV- 18 in keeping with previous studies (Lee et al., 2001; Motlagh et al., 2007; Giuliani et al., 2008). Furthermore, we detected HPV- 45 and HPV- 31, which have not been previously reported in patients with colorectal carcinoma in Iran but have been recognized as high- risk types in malignant diseases (Yahyapour et al., 2013). In comparison with this study, Damin et al. (2007), Chen et al. (2012) and Burnett-Hartman et al. (2013) found HPV- 16 as the most frequently detected HPV type in patients with CRC. Therefore, this study supports the hypothesis that expresses the different frequency patterns of HPV infection according to geographical variations and ethnicity of the populations (Jung et al., 2004; Chansaenroj et al., 2012; Miąsko et al., 2012).

According to Table 3, analysis of medical data in infected patients with CRC in compare to non- infected patients with CRC explained a possible association between high- risk types of HPV infections and accession to high- grade malignant tumors. The findings of Da Costa

et al. (2002), Gillison and shah (2003), Chen et al. (2012), Hamid and Mustafa (2012), Ghasemian et al. (2013), Zandberg et al. (2013) in comparison with results from Motlagh et al. (2007), Giuliani et al. (2008) investigate the ability of HPV infection to induce advanced stages of cancer in different malignancies. Furthermore, this study highlights the impact of high- risk types of HPV infection in distant metastasis and malignant lymph nodes invasion in compare with non-infected patients, which have been previously reported in several investigations (Chen et al., 1993; Gillison and shah, 2003; Bognar et al., 2008; McHugh et al., 2009; Zandberg et al., 2013).

Gillison and Shah (2003) indicated that HPV associated malignancies would occur at anatomic sub-sites of exposure by direct contact, since there is no viremic phase in the pathogenesis of HPV, so the infection is not widely disseminated in the body. On the other hand, Bodaghi et al. (2005) reported that HPV infection in the tumor tissues which obtained from the cecum and ascending is as common as in the tissues obtained from rectosigmoid locations and this indicated the HPV infection might not be a result of the direct spread from anogenital sites, also Chen et al. (2012) found that the transmission of the HPV to the colorectal tissues might occur through blood circulation. Since higher frequency of HPV infection in cecal and ascending tissues has been determined in compare to frequency of infection in rectosigmoid tissues, this study supports the hypothesis that investigated HPV infection in colon and rectum might not occurs through direct infection.

Although an appropriate sample size was selected for this study, but HPV infection rate was less than our prediction and we found no certain evidence to support the aetiological role of HPV infection in colorectal carcinoma. On the other hand, medical records indicated that HPV infection, particularly with high- risk types might be associated with high- grade malignant tumors, progression of colorectal carcinoma and metastasis. This study shows an almost different frequency of HPV genotypes in patients with colorectal cancer in Iran and since HPV vaccines are limited to a few types of virus, it is necessary to find the most frequent types of the virus in different population to optimization the current vaccines according to geographical prevalence of the virus's types. Therefore, using cohort studies in different geographical zones to screening the frequency of HPV genotypes and its association with colorectal carcinoma might increase the efficacy of the current vaccines.

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