Lack of association between human papillomavirus infection and colorectal cancer

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Abstract

Introduction: Colorectal cancer is the third leading cause of cancer-related deaths worldwide, with nearly one million new cases identified annually. Different factors might cause colorectal cancer, one of the most prevalent cancers among both men and women. Viral aetiology in cancerous malignancies is a very important issue and so far a number of viral strains have been identified as tumour oncogene viruses. Viral infections, such as human papillomavirus (HPV), have recently been suggested as a risk factor for colorectal cancer. However, the aetiology of the disease is still unknown.

Aim: To assessed the association between HPV infection and colorectal cancer.

Material and methods: In this study, 50 cancer tissue samples and 50 samples without colon cancer were studied in order to identify HPV through polymerase chain reaction (PCR). Of 42 adenocarcinomas, 10 were well differentiated, 30 moderated differentiated, and 2 were poorly differentiated. DNA extraction was verified by beta globin gene amplification; specific PCR was carried out based on HPV L1 consensus primers MY09/MY11.

Results: HPV DNA was not identified in any of the normal, adenocarcinoma, or adenoma samples.

Conclusions: In contrast with previous studies, the current research failed to establish a relationship between HPV infection and the incidence of colon cancer. Considering the existing inconsistencies, it is recommended that further studies be conducted with larger sample size.

Introduction

Colorectal cancer is the third cause of cancer death worldwide [1]. Smoking, alcohol and red meat consumption, low dietary intake of vegetables, obesity, age, family history, and physical inactivity can increase the risk of colon cancer. Colorectal cancer originates from the epithelial cells in the colon and rectum. The ability of human colon cells to rapidly replicate leads to the mutation in epithelial cells of the colon and rectum and eventually carcinogenesis [2]. About 3–5% of colorectal cancers have been proven to be related with mutations in specific genes [3]. The accumulation of mutations in oncogenes and tumour suppressor genes alters cell morphology and causes hyperproliferation in abnormal cells, and thus neoplasia [4]. Adenomatous polyps are benign lesions of the colon and rectum that may develop into cancer [5]. The human intestine is a nutrient-rich environment where 500 bacterial species and pathogenic and non-pathogenic viruses grow [6]. Due to the large microbial populations in these parts of the human body and the role of infectious agents as risk factors for different cancers, researchers throughout the world have recently evaluated the relationship between various infectious agents and the incidence of colorectal cancer.

About 20% of cancers are estimated to be related with infectious agents. Numerous DNA viruses, including Hepatitis B virus (HBV), Epstein-Barr virus (EBV), Kaposi sarcoma herpesvirus (KSHV), and Human papillomavirus (HPV), have strong relationships with human neoplasm [7, 8]. Research on the relationship between HPV and human cancers increased after Zar Hausen revealed the presence of HPV DNA molecules in patients

with cervical cancer in 1983–1984 [9]. Despite the evident relationship of HPV infection with cervical cancer and anogenital tumours, the possible associations between HPV and other malignancies such as respiratory system, gastrointestinal, breast, and colorectal cancers are yet to be clarified [8–10].

While several reports have indicated the role of HPV DNA in colorectal tumours [12–14], several others have suggested the absence of the agent in colorectal cancer [15, 16]. Although knowledge about the molecular pathogenesis of colorectal cancer has improved over recent decades, the existing inconsistencies mean that the aetiology of the disease remains ambiguous.

Aim

The present study employed a precise method, polymerase chain reaction (PCR), to examine the presence of HPV DNA in colorectal tumours. It then sought to determine any significant relationship between the presence of the virus and the incidence of colorectal cancer.

Material and methods

Tissue sample selection

All specimens were provided by the Pathology Department of Imam Khomeini Hospital (Tehran-Iran). Specimens were investigated by a pathologist and then used for DNA extraction and PCR analysis. The specimens included in the study consisted of 50 formalin-fixed tissues of colorectal cancer including 8 colorectal adenomas, 42 colorectal adenocarcinomas, and 50 normal tissues with no malignancy. Of the 42 adenocarcinomas, 10 were well differentiated, 30 were moderated differentiated, and 2 were poorly differentiated.

Genomic DNA extraction

Total cellular DNA was extracted from samples using a General Genomic Extraction Kit according to the manufacturer's instructions. This kit was newly developed by the first author of the paper. Briefly, 0.05 g of chopped tissues were mixed with lysis buffer (Solution A) and 30 µl proteinase K (20 mg/ml) in 1.5 ml microtubes and incubated at 65°C for 3 h. Microtubes were inverted for 15 min for good solution of the crushed tissues with the buffer. Six hundred µl was buffer gbindin o of (solution B) added and centrifuged at 12,000 rpm for 5 min. The upper aqueous phase was separated without disturbing the interphase. This step was repeated once again. The aqueous phase in each tube was transferred to a new 1.5 ml microcentrifuge tube. Six hundred µl of cold Precipitation Buffer (Solution C) was added and inverted for 20 min. The resultant mixture was centrifuged at 12,000 rpm for 10 min and the upper aqueous phase was removed. The DNA pellet was washed with cold washing buffer (solution D) followed by 15 min of mild inversion at room temperature and centrifugation at 12,000 rpm at 4°C for 10 min. Washed DNA pellets were dried by leaving the tubes at 37°C for 40 min. DNA sample was dissolved in 50 µl solvent buffer. Genomic DNA purity was assessed with a NanoDrop™ ND-2000 spectrophotometer and calculated by the ratio of the DNA optical density (OD 260) to the protein optical density (OD 280). Genomic DNA yield was calculated from DNA optical density (OD 260) for clean DNA samples. The quality of extracted DNA was analysed by electrophoresis in 1% agarose gel stained with gel red.

Verification of DNA extraction

All samples were examined for DNA integrity using amplification of the β-globin. Sequences of primers were PC04: 5' CAA CTT CAT CCA CGT TCA CC 3' and GH20: 5' GAA GAG CCA AGG ACA GGT AC 3'. Polymerase chain reactions were carried out in a final volume of 25 µl containing 12.5 µl of Amplicon master mix, 0.5 µl of forward and reverse primers, and 1 µl of DNA template. Amplification was carried out in a thermal cycler (Biorad). After an initial denaturation step at 95°C for 3 min, 45 cycles were programmed as follows: denaturation step at 95°C for 30 s, annealing step at 53°C for 40 s, primer extension at 72°C for 40 s, and final extension step at 72°C for 5 min. Polymerase chain reaction products were determined by visualisation of amplicons on 2% agarose gels stained with gel red [16].

Polymerase chain reaction amplification

Specific PCR was carried out based on HPV L1 consensus primers MY09/MY11 (MY09: 5'-CGTCCMARRGGAWACTGATC-3', and MY11: 5'-GCMCAGGGWCATAAYAATGG-3') [16]. The PCR amplification was performed in a 25 µl reaction volume containing 12.5 µl of Amplicon master mix, 0.5 µl of forward and reverse primers, and 5 µl of each genomic DNA sample. The PCR program was performed as follows: pre-denaturation at 95°C for 3 min, 1 cycle; denaturation at 95°C for 30 s, annealing at 63°C for 40 s, extension at 72°C for 40 s, 45 cycles; and post-extension at 72°C for 5 min, 1 cycle. At the end of amplification, 5 µl of the PCR product was analysed on 2% agarose gel. The resultant product was expected to be a 450-bp fragment.

Results

The mean age of the subjects was 52 years (youngest 16 and oldest 79). The tissues studied included 42 colorectal adenocarcinoma and 8 colorectal adenoma tissues. The adenocarcinoma tissues were categorised in one of three stages; poorly, moderately, and

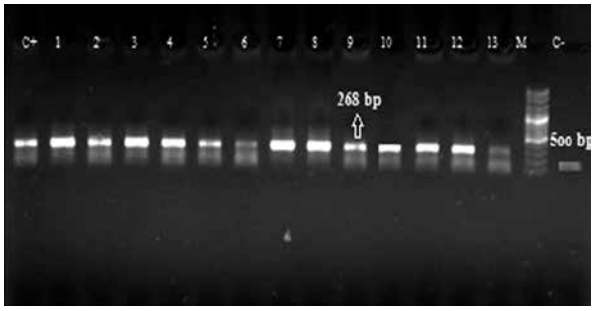

Figure 1. PCR of the β-globin gene was used as an internal control for DNA extraction. Lane C^+ – positive control; lanes 1–13 – PCR products of β-globin gene (268 bp feragment), M – 100 base pairs DNA Ladder, C^- – negative control

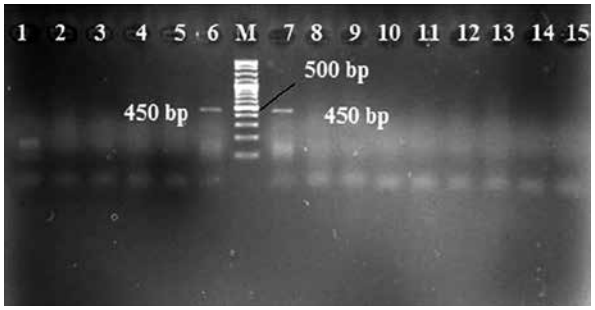

Figure 2. Profile of specific PCR for detection of HPV gene (450-bp PCR product) on 2% agarose gel. Lanes 1–5 – no detection of PCR products in some normal tissues, 6 – positive control, M – 100 base pairs DNA Ladder, lane 7 – positive control, lane 8 – negative control, lanes 9–15 – no detection of PCR products in some colorectal cancer tissues

well differentiated. To extract DNA from the tissues, β-globin primer was used to conduct a PCR. After verification of the 268-bp fragment, samples were selected for the specific PCR. Given the quality and quantity of the extracted DNA, 268-bp fragments proliferated in all samples (including normal and cancerous tissues) (Figure 1). Then, a specific PCR test was used to evaluate the viral DNA. The results showed that 450-bp fragments do not proliferate in any cases of colorectal cancer tissues (Figure 2).

Discussion

Colorectal cancer is the fourth most common cancer in men and the third in women and has an annual incidence rate of about one million [17]. Oncogenic HPV contributes to benign and malignant cervical, vulvar, vaginal, anal, and penis lesions [18–20]. The development of colorectal cancer is a multifactorial, heterogeneous process in which environmental, lifestyle, and genetic factors are involved [21]. About 95% of colorectal cancers are adenocarcinoma originating from previously developed adenomas [21]. Furthermore, several molecular abnormalities, e.g. oncogenic mutations (like k-ras mutations), deactivation of tumour suppressor genes including P53, APC, and DCC, disturbances in DNA methylation, and microsatellite instability, have been reported in colorectal carcinoma. The majority of these genetic changes can be effective in altering normal mucosa to adenoma and finally to carcinoma [21–23]. Genetic instability, cell immortalisation, high cell proliferation, and resistance to apoptosis are the main characteristics of malignant neoplasm, some of which are essential for viral replication.

DNA tumour viruses use different strategies to control key cellular processes, such as cell proliferation, cellular signalling and differentiation, and apoptosis [8]. Oncogenic potential of HPV results from the activity of two early viral oncogenes called E6 and E7 [24, 25]. HPV E7 is also an oncoprotein involved in the induction of genomic instability, i.e. HPV E6 and E7 induce protein 53 (P53) and retinoblastoma protein (pRb, an important tumour suppressor) degradation, respectively [8]. These two tumour suppressor proteins take part in cell cycle progression, DNA repair, apoptosis, cellular differentiation, and chromatin remodelling [24]. The mentioned molecular mechanisms imply that HPV infection can damage critical cellular processes during the development of cancer. Studies on the possible role of HPV in cancers were initiated in the 1970s. The virus is currently considered responsible for over 95% of cervical cancers throughout the world [7, 9]. However, the relationship between HPV infection and colorectal cancer is still controversial.

Despite the identification of HPV in colorectal adenocarcinoma tissue [14, 26, 27], PCR did not detect HPV DNA in adenocarcinoma tissue evaluated in the current study. Our findings are in contrast with other research in this field [15, 16, 28–30]. Such inconsistencies can be justified by false-positive results due to contamination during testing and differences in sample size, sensitivity of employed techniques, geographic diversity of the study populations, and HPV prevalence in the study geographical area. Since molecular methods are highly sensitive and viral infections can easily spread at any stage of testing (e.g. biopsy, tissue section preparation, DNA extraction, and PCR), controlling viral contamination during examinations is crucial to ensure reliable results. The basis of all analyses is the type of studied tissue. Most studies use paraffined blocks that should be separately made using sterilised surgical blades and under sterile conditions. In other words, preventing

contamination during microtomy requires individual sterile blades and thorough cleaning of the device for each tissue slice. Failure to take the above-mentioned measures may spread infection from one tissue to other samples and produce false-positive results. Similar facts have also been suggested by other researchers [28, 29].

An advantage of the current study was using fresh tissue instead of paraffined blocks. Moreover, the samples were frozen at –80°C immediately after histologic examination. The intervals between sampling and freezing and DNA extraction are crucial since shorter intervals increase the chance of identification and survival of HPV DNA [29]. In order for DNA extraction, each section was prepared by utilising a scalpel blade, and disposable gloves and plates. Moreover, one positive and one negative control were considered for every 20 samples in PCR. Sterile distilled water was used as the negative control. The positive controls did not indicate any contamination during the PCR. On the other hand, the number of samples was appropriate and the applied technique had a desirable power in identifying HPV DNA. In fact, MY09/MY11 primer can identify a wide range of high-risk HPV (6, 11, 16, 18, 31, 33, 35, 39, 40, 45, 51–59) and has been previously employed in HPV DNA identification [5, 16, 26, 27, 30–32].

Another noteworthy factor is the prevalence of HPV in the study population. Likewise, Yuvuzer *et al.* reported the low prevalence of HPV in Turkey [31]. Limited available information suggests that the prevalence of the virus is not high in Iran (5.5–29.0%) [33, 34]. Moreover, the age of the study population should not be ignored. For instance, since the mean age of our participants was 52 years, so we actually evaluated a low-risk population.

Conclusions

According to inconsistent findings about the relationship between HPV and colorectal cancer, larger studies with proper sampling and the application of PCR are recommended. Moreover, with HPV being sexually transmitted, high-risk sexual factors such as the number of sexual partners, age at first intercourse, and history of anal sex (none of which have been evaluated in previous research) have to be included in future studies.

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