Human Papilloma Virus and Esophageal Squamous Cell Carcinoma

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Abstract- Human papillomavirus (HPV) has also been suggested as an etiology of esophageal squamous cell carcinoma (SCC). The aim of this study was to investigate the prevalence of HPV infection in esophageal SCCs in our region with strict contamination control to prevent false positive results. Thirty cases of esophageal squamous cell carcinomas were chosen by simple random selection in a period of two years. PCR for target sequence of HPV L1 gene was performed on nucleic acid extracted from samples by means of GP5+/GP6+ primers. All tissue samples in both case and control groups were negative for HPV-DNA. Although the number of cases in this study was limited, the contribution of HPV in the substantial number of esophageal SCCs in our region is unlikely.

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Introduction

Squamous cell carcinoma (SCC) is one of the common malignancies of esophagus (1,2). Because the tumor is usually advanced at the time of diagnosis, it causes high morbidity and mortality. Prevalence of esophageal SCC is different in distinct geographic and cultural conditions, and esophageal SCC is uncommon in United State and Western Europe in contrast to china ,Latin America , middle Asia and Iran (2,3). Genetic and environmental factors play a role in the pathogenesis of esophageal SCC; multiple environmental etiologies have been suggested for esophageal SCC, for example smoking, alcohol consumption, diet and caustic agents (4,5). Human papillomavirus (HPV) has also been suggested as an etiology (6).

Human papilloma virus (HPV) is an oncogenic, double-stranded, DNA-virus that infects skin and mucosa. Usually associated with benign papillomas, mucosal HPV Infection is one of the most common sexually transmitted infections (7). Moreover, mucosal infection with high-risk types, such as HPV 16 and 18, can rarely progress to dysplasia and cancer, a process that takes a long time to occur (7,8). Viral oncoproteins E6 and E7 target p53 and retinoblastoma protein (RB), interfere with cell cycle, and initiate malignant transformation. SCC and adenocarcinoma of the uterine cervix, vagina, and vulva; and SCC of oropharynx are among the tumors caused by HPV. Oropharyngeal SCC, when associated with HPV, shows better prognosis in contrast to its non-HPV related counterpart (9,10). If a relation exists between HPV and SCC in esophagus, HPV vaccination can be utilized for prevention of this tumor; meanwhile, HPV detection can be valuable for determination of prognosis.

The aim of this study was to investigate the prevalence of HPV infection in esophageal SCCs in our region with strict contamination control to prevent false positive results.

Materials and Methods

In a previous study, prevalence of HPV infection in normal esophagus and esophageal squamous cell carcinoma were about 0% (q) and 23% (p) respectively. (11) Therefore, sample size of this case control study was calculated for comparison of qualitative characteristics analysis. Consequently, thirty cases of esophageal squamous cell carcinomas were chosen by simple random selection in a period of two years from 2007 to 2009, by searching the admission database of Cancer Institute, Tehran University of Medical Sciences, paraffin block containing tumor tissue was available in each case. Thirty samples of gastroesophagectomy for gastric adenocarcinoma with involved esophageal margin were also selected by the same method as a

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control group; likewise, paraffin block containing normal esophageal tissue was available in each sample. Patients are admitted to this referral center from all regions of Iran, representing a wide range of socioeconomic levels. H&E stained slide of each paraffin block was reviewed for the presence of tumor in case group and the absence of tumor in control group. Demographic and pathologic data, as well as PCR results of case and control groups, were analyzed by SPSS version 12.0 (SPSS Inc).

Extraction

From each paraffin block, two 5μM sections were prepared using a new disposable blade; the sections were transferred to a 1.5mL Eppendorf tube by a disposable applicator. The tubes were briefly centrifuged to make a paraffin embedded tissue pellet. Then tissue was deparafinated by Xylene and nucleic acid was extracted using Roche High Pure RNA Paraffin Kit (Roche Diagnostics, Indianapolis, IN) as instructed by the manufacturer without going through the DNA elimination procedure. Eluted DNA was stored in a -20ºC freezer until HPV detection was performed.

Polymerase chain reaction (PCR)

To ensure the validity of the purification procedure and exclusion of PCR inhibition, Homo sapiens hydroxymethylbilane synthase (HMBS) was amplified as endogenous internal control was amplified as described previously (12,13).

The PCR for target sequence of HPV L1 gene was performed in 20μL reaction containing 0.5μM of each forward and reverse GP5+/GP6+ primers, and 10μL SYBR Premix Ex Taq (Takara Bio, Ostu, Shiga, Japan). The PCR method for HPV L1 region was validated by means of six HPV 16 samples, proved by sequencing, from uterine cervix squamous cell carcinoma samples. The touch-down PCR was employed for its superior sensitivity. (14) The PCR conditions were: initial denaturation at 94 for 0.5 min; then 45 cycles as follows: 95°C for 10 seconds; first initial 20 cycles at 60°C to 50°C for 20 seconds (with 0.5°C decrease in each cycle followed by 25 cycles of 50°C for 20 seconds; and 72°C for 34 seconds. The Real-time acquisition was performed during annealing phase on FAM channel. The touchdown annealing phase composed of 20 cycles of 60°C to 50°C with 0.5°C decrease in each cycle. Finally, a 5 min terminal extension was performed followed by melting analysis of PCR product. Target melting temperature, and the sequence of primers are shown in table 1.

A rotor-gene 300 real-time thermal-cycler (Corbett Research, Mortlake, Australia) was used for internal-control and target amplification reactions. Positive control of HPV type 16 was included in each run.

Table 1. Sequence of the primer utilized in this study

Primer	Sequence 5′-3′	Melting Temperature (^{o}C)
GP5+/GP6+	TTTGTTACTGTGGTAGATACTAC GAAAAATAAACTGTAAATCATATTC	80
HMBS	GCCTGCAGTTTGAAATCAGTG CGGGACGGGCTTTAGCTA	84

Result

There were 30 patients in the case group, 14 of which were male, and the rest were female. Average age of the patients in the case group was 59.6 and ranged from 30 years to 83 years. There were 30 patients in the control group, 16 of which were male, and the rest were female. Average age of the patients in the control group was 62 and ranged from 41 years to 82 years.

All tumors of the case group were squamous cell carcinomas with 33% grade I, 50% grade II and the rest were grade III. Tumor depth of invasion, tumor location and lymph node involvement are summarized in table 2. All tissue samples in both groups were positive for HMBS internal control gene and negative for HPV L1 gene.

Discussion

A causative role for HPV in esophageal SCC has been implied by some authors from Iran, China, latin America and Japan (11,15-18). Meanwhile, a number of reports from other countries have questioned a substantial relation (19,20). Therefore, there is no consensus agreement on this issue. The reason might be that; not only geographic distribution of HPV infection and its genotypes are varied, but also the sensitivity and specificity of HPV detection methods are different (21, 22). Secondary infection of the neoplastic squamous epithelium has also been suggested by some authors (19). Most of the studies that suggested a role for HPV in esophageal SSC were based on polymerase chain reaction (PCR). Being extremely sensitive method, conventional-PCR and specially nested-PCR are prone

to contamination by post-PCR products that cause false positive results.

Table 2. Pathological and clinical information of the case group

		Frequency	Percentage %
Grade of tumor	**Poorly Differentiated**	5	16.7%
	Moderately Differentiated	15	50.0%
	Well Differentiated	10	33.3%
Site of tumor	**Upper Third**	7	23.3%
	Middle Third	12	40.0%
	Lower Third	11	36.7%
Lymph node involvement	**Yes**	12	40.0%
	No	10	33.3%
	Cannot be assessed	8	26.7%
Depth of invasion	**Lamina propria or submucosa**	3	10.0%
	Muscularis propria	5	16.7%
	Adventitia	22	73.3%
Metastasis	**Yes**	5	16.7%
	Cannot be assessed	25	83.3%

The advantage of this study was employment of real-time PCR. In real-time PCR, in contrast to conventional PCR, the estimation of the relative amount of target sequence is possible by comparing threshold cycles (Ct) of the target and internal control genes. Because malignant tumors are clonal, if there is a causative virus, it is expected to be present at high amounts. In other words, the causative virus ought to be present in many tumor cell nuclei (8,23). Evidence from previous studies suggests that head and neck carcinomas have been no exception to this (10). Therefore, when there are small amounts of viral DNA in a tumor, contamination or secondary infection may be more likely than a real cause-effect phenomenon (19).

Moreover, in conventional PCR where products are detected after completion of the amplification phase, contamination is always a concern (24). Another advantage of the real-time PCR method is that amplification and detection are performed in the same time, and the system is closed. Therefore in the latter, the risk of PCR contamination with amplicons is minimal, which in turn decreases false positive rate. This may be the main reason behind the fact that recent reports question a causative role for HPV in esophageal carcinomas (19-21).

A limitation of this study, common to all studies that use L1 amplification, is that in the minority of cases HPV integrates to host genome and parts of L1 might be deleted. Therefore, although HPV is present, it is not amplified, and the test becomes false negative (13). A number of these cases is small, and it is unlikely that it highly affected the results of the current study (22). Moreover, employment of formalin fixed paraffin embedded tissue is another limitation of this study. However, internal control amplifications were used to minimize the effect of formalin fixation on the results of this study.

In conclusion, although the number of cases in this study was limited, the contribution of HPV in a substantial number of esophageal SCCs in our region is unlikely.

Take-home message

A causative role for HPV in esophageal SCC has been implied by some authors. Meanwhile, a number of other reports have questioned a substantial relation. The contribution of HPV in a substantial number of esophageal SCCs in our region is unlikely.

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