Original Article

Frequency and genotype distribution of high risk human papillomavirus in esophageal squamous cell carcinoma

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Abstract

Introduction: Esophageal squamous cell carcinoma (ESCC) is a dismal disease exhibiting striking geographical differences in its incidence. It is multifactorial in origin. Among infectious agents, human papillomavirus (HPV) was introduced as a possible causative agent in the development of ESCC in 1982. Subsequent studies using various methods have confirmed the presence of HPV in ESCC. We aimed to determine the frequency of HPV in ESCC in northwest Pakistan which is part of high risk belt for this disease.

Methodology: This study was conducted on two hundred and forty-three (243) diagnosed cases of ESSC at two tertiary care hospitals of Peshawar, Pakistan, from 2011 to 2016. DNA was extracted from all specimens. Polymerase chain reaction (PCR) was used to check the quality of DNA using β-globin primers and frequency and genotypes of HPV using HPV general primers and type-specific primers respectively. HPV and its genotypes were confirmed through the sequencing of a few selected cases.

Results: Two hundred and three (203) tissue specimens had adequate DNA and were further analyzed. HPV positivity with general primers alone was 15.7% (32/203). Using HPV general primers and type-specific primers (HPV 16 or HPV 18), the overall positivity of HPV was 31% (63/203). For type-specific primers, frequency of HPV types 16 and 18 was 20.19% (41) and 7.8% (16) respectively where 6 cases were positive for both HPV 16 and 18.

Conclusions: The overall high prevalence of HPV indicates it as a possible risk factor for ESSC.

Key words: Esophageal squamous cell carcinoma; human papillomavirus; infectious agents.

Introduction

Esophageal cancer (EC) is one of the major global medical and public health challenges because of its aggressive nature, late presentation and consistently high mortality rate. Esophageal cancer (EC) has been ranked the seventh most frequent cancer worldwide with 572,000 new cases per year. It is the sixth most common cause of mortality due to cancer with 509,000 deaths per year and an estimated death rate of 1/20 cancer deaths in 2018 [1]. Incidence rates according to the World Health Organization are highest in China and lowest in western Africa. [2] Globally, Squamous cell carcinoma is the most common type of esophageal cancer despite the recent rise in the incidence of adenocarcinoma subtype in developed countries [5]. According to Globocan 2012, Esophageal Cancer in Pakistan is 9th and 5th most common cancer in males and females, respectively [3]. In Pakistan, a national scale cancer registry does not exist [4]. Previous data from the Punjab cancer registry (only cancer registry based on population) listed esophageal cancer as the 4th most common cancer in adults [3]. Karachi cancer registry listed esophageal cancer as the most common cancer in Karachi [5,6]. Quetta cancer registry reported that esophageal cancer is very common among both genders [7]. CENAR, a cancer-treating hospital in Baluchistan reported it to be the 3rd most common cancer in their practice. According to unpublished data from the Dow University of health sciences, Esophageal Cancer is 3rd most common cancer in females and 5th in males [3]. All these data suggest that Pakistan is a country with high esophageal cancer burden but a large number of cases remain unreported and available data is only the tip of the ice berg. The available data is also from major cities only harboring the tertiary health care centers. An alarming fact is the complete absence of data regarding

the frequency and distribution of esophageal cancer from over 80 cities of Pakistan [3]. Khyber Pakhtunkhwa shares a long border with Afghanistan, and has a high prevalence of esophageal cancer [8]. As the border is extremely busy for cross border traveling, therefore it may also affect the esophageal cancer statistics in Pakistan.

Risk factors for ESCC in high endemic developing areas are tobacco smoking, alcohol consumption, processed red meat and nutritional deficiencies of fruits and vegetables, exposure to polycyclic aromatic hydrocarbons, nitrosamines as well as biological risk factors including bacteria, fungi and some viruses [9]. Environmental factors have gained importance because of marked ethnic, geographical and cultural variations. Several risk factors including dietary and cultural habits, nutritional deficiencies, infectious agents are suggested as possible causes [10]. All these lead to chronic mucosal injury, and probably synergistically augmenting the effects of each other leading to cancer, therefore esophageal carcinoma is multifactorial. The overall rate of HPV in esophageal squamous cell carcinoma is the most well studied yet a debatable issue, facing a lot of controversies. A number of studies have been conducted to resolve these controversies using different populations, techniques and controls, but failed. [11-14]. Due to the introduction of preventive vaccines against human papillomavirus, the need to clarify the association of HPV with other cancers besides cervical cancer has increased and Esophageal Cancer may be one such target [11].

Syrjanen first suggested the association of HPV and ESCC in 1982 as he found morphologically similar HPV induced lesions in the genital tract and ESCC [15]. Several studies since then have tried to confirm the possible role of HPV in cervical cancer, however, producing conflicting results in extragenital cancers [16]. Human papillomaviruses (HPVs) are small, non-enveloped, double-stranded circular DNA viruses with 221 HPV types [17]. They are further divided into two groups, high-risk (HR) and low-risk HPVs. HR-HPV type 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59 are associated with 99% of cervical cancers, 70-90% of anal and vaginal cancers, 47% of penile cancers, 40% of vulvar cancers and 25-30% of oropharyngeal cancers [18]. Recently HPV is accepted as a risk factor and indicator of better survival in head and neck squamous cell carcinoma [19]. HPV can infect the esophagus in the same way as oral cavity, tonsils and pharynx [20]. This is due to the fact that the esophagus is anatomically continuous with the upper aerodigestive tract. There are also histological similarities between the esophagus and head and neck squamous epithelia. Common histopathological subtypes of esophageal cancer in the esophagus and head and neck is squamous cell carcinoma suggest that ESCC may be associated with HPV [21]. However different studies have shown conflicting results regarding the prevalence of HPV in ESCC patients depending upon geographic area, ethnic groups, tissue samples and detection methods used [20]. We tried to analyze the presence of HPV in ESCC in a genetically distinct population of Pakistan having unique social and environmental factors as they are not investigated. This is the first study to our knowledge detecting the frequency of HPV in ESCC using PCR.

Methodology

Study Population and Samples

This descriptive study was conducted within two tertiary care hospitals, that is, Northwest General Hospital and Research Centre and Khyber teaching hospital Peshawar, Pakistan from June 2011 to June 2016. The study was approved by the ethical committee of Khyber Medical University and Northwest General Hospital. Written informed consent was obtained from the participants.

All those patients with a confirmed diagnosis of esophageal squamous cell carcinoma were included in the study. Similarly, patients with sufficient biopsy tissue for extraction of DNA were included. Patients with esophageal adenocarcinoma, insufficient tissue or those who were not willing to participate were excluded from the study. Furthermore, patients with dysplasia or suspicious/suggestive diagnosis of ESCC were also excluded.

Two hundred and forty-three (243) formalin fixed paraffin embedded (FFPE) specimens of ESCC were analyzed. Using rotary microtome, 4 micron thick sections were cut from the specimens, stained with Hematoxylin and Eosin and examined under a microscope by two pathologists to confirm the presence of ESCC.

Deoxyribonucleic acid (DNA) extraction

Tissue weighing 25 µg was taken from FFPE specimens with a surgical blade. The blade was changed for each case. Tissue was cut into small pieces. Dewaxing was done twice by incubating the tissue for one hour in pure xylene. Washing and dehydration were done twice with pure ethanol. DNA was extracted using the Qiagen QIAamp DNA Mini kit (Valencia, CA) according to the manufacturer's instruction. A 20 µL of proteinase K and 180 µL buffer ATL was added, mixed by vortexing, and incubated at 56 °C (overnight) using

a water bath. After 24 hours, the temperature of the water bath was increased to 90 °C for 1 hour or till the tissue was completely lysed. After brief centrifugation, 200 µL Buffer AL was added, mixed by vortexing for 15s, and incubated at 70 °C for 10 minutes. After brief centrifugation, 200 µL ethanol (96-100%) was added and vortexed for 15 seconds. The lysate was briefly centrifuged and carefully applied to the mini spin column in a 2 mL collection tube. It was then centrifuged at (8000 rpm) for 1 minute. The mini spin column was placed in a 2 mL collection tube, and the tube with the filtrate was discarded. The whole process was repeated with 600 µL buffer AW1 and AW2. After discarding, the cap was closed and centrifugation was done at full speed of 14,000 rpm for 3 minutes. The mini spin column was now placed in a clean 1.5 mL microcentrifuge tube. And 80-100 µL buffer AE was added. It was incubated at room temperature for 5-10 minutes, and then centrifuged at (8000 rpm) for 1 minute. The step was repeated and DNA thus eluted was stored in -20 °C freezer till used for HPV detection. To avoid cross-contamination, few processes were applied including powder-free gloves, disposable filter tips and sterile Eppendorf tubes. Microtome, surfaces and biosafety cabinet were also cleaned periodically with 70% ethanol.

PCR analysis

DNA extracted from all samples was subjected to PCR amplification. In order to ensure the quality and integrity of DNA beta-globin gene, specific primers were used in each case. For the presence of HPV, a pair of HPV general primers GP5+/GP6+ were used to amplify L1 region of HPV. HPV16 and 18 were tested in all samples using type-specific primers. The sequences of forward and reverse beta-globin gene primers, HPV general primers and HPV 16, 18 specific primers set with their annealing temperatures and product sizes are shown in Table 1.

PCR was carried out with a 20 µL reaction mixture containing 0.5 µL of each GP5+/GP6+ primers, 10 µL of master mix and 2.5 µL of DNA template. PCR water was used as a negative control (all reactants minus

target DNA) and HPV 16 or 18 positive samples confirmed by sequencing were used as the control in every run of all PCR reactions using a thermocycler. PCR conditions for HPV were: denaturation at 95°C for 5 minutes, then 35 cycles as follows: 95°C for 40 seconds. Annealing was done at 50°C and extension at 72°C first for 30 seconds and then for 5 minutes. PCR tubes were transferred to a refrigerator for later use in detection and analysis of the PCR products on 1.5% agarose gel electrophoresis with ethidium bromide staining. Patients were considered positive for HPV after successful PCR amplification twice.

HPV type determination: For HPV genotyping, type-specific primers against HPV type 16 and 18 were used. PCR conditions were the same as for HPV general primers with difference only in annealing temperatures. Multiplex PCR with primers 6, 11, 31, 33 and 58 was used for cases negative for HPV 16 and 18.

The PCR products from selected HPV positive cases and HPV 16 and 18 positive cases were employed for sequencing. DNA sequences obtained were compared with available DNA sequences in Gene bank through the basic local alignment search tool (BLAST).

Statistical analysis

Software IBM SPSS Statistics for Windows, version 22, was used for data analysis. HPV and its types percentage and frequencies were calculated.

Results

Quality of DNA as detected by β-globin PCR was good in 203 out of 243 cases. All 203 cases having clearly differentiated DNA bands of 268 bp as defined by β globin PCR were analyzed further. 40 cases having no bands on the gel were excluded and were not processed further to detect HPV or its types. To confirm the PCR products of interest, all PCR processes were carried out twice. The products of the first PCR were utilized as a template for the second PCR. Table 2 summarizes the results of every PCR process for 203 cases. All results shown are those of the second PCR.

Using both general primer set (GP5+/GP6+) and type-specific primers (HPV 16 or HPV 18), overall

Table 1. Primers used in PCR amplification of HPV DNA with respective amplicon size.

Primer type	Forward primer	Reverse primer	Annealing Temp	Amplicon Size bp
Beta Globin	AAGAGCCAAGGARCAGGTAC	CAACTTCATCCACGTTCACC	60° C	268
HPV General primer	GP5+: TTTGTTACTGTGGTAGATACTC	GP6+: GAAAAATAAABGTAAGTCATTC	50 °C	150
HPV16	TTAGGCAGEACTTGGCCAACCA	TAATCCGTCCTTTGTGTGAGCT	58 °C	207
HPV18	TCCGTGGTGTGCATCCCAGCAG	CACTTGTGCACATTGTGGACC	48 °C	274

positivity of HPV was 31% (63/203 cases). HPV positivity with general primer set (GP5+/GP6+) only was 15.7% (32/203 cases) as indicated by 150 bp PCR product on 1.5 % agarose gel. Out of 32 HPV positive cases, 10 cases were HPV16 positive, 8 cases were HPV 18 positive, 2 cases had co-infection and were positive for both HPV 16 and HPV 18. Remaining 12 HPV positive cases were negative for type-specific primers (HPV type 16 or 18). They were subjected to multiplex PCR containing primers for HPV types 6, 11, 31, 33 and 58. The process carried out thrice did not show any positive results. Out of the remaining 31 HPV L1 negative cases, 25 HPV L1 negative samples were detected harboring HPV 16 and 6 samples were harboring HPV 18 by using type-specific primers.

While using type-specific primers only, HPV 16 was positive in 20 % (41/203 cases) as indicated by the presence of a band of the correct size (207 bp) and 7.8% (16/203 cases) were positive for HPV 18 with amplification size of 274 bp.

Discussion

ESCC is one of the deadliest cancers with a very poor prognosis. Despite recent advances, still the cure rate and patient survival are low [22]. Recently, ESCC complicated with HPV has been extensively studied worldwide but the results are variable [23]. Methods used for the detection of HPV in ESCC patients include Southern blotting, dot blot hybridization, HPV L1 serology, *in situ* hybridization and Polymerase chain reaction. Different reviews have reported that the reason for variable results reporting HPV in ESCC may be explained at least partially by the evaluation of the detection method used [13,24]. Conventional PCR for the detection of HPV in tumors is known to be incomparably the most sensitive and rapid method among the available techniques [25]. It is now being used for more than 20 years having the benefit of providing type-specific diagnosis [26]. Advantage of using PCR as a detection technique is its ability to detect HPV well below one viral copy of genome per cell [26]. The current study was designed to determine the frequency and type-specific distribution of HPV in ESCC using PCR. Although PCR is an extremely sensitive and specific method but it is prone to high false-positive results [27,28].

Our data shows that the overall frequency of HPV is 31% (63/203 cases) by adding the prevalence of consensus AND type-specific primers. PCR with type-specific primers of HPV 16 and HPV 18 amplify type-specific regions E6/E7 of specific HPV types and are therefore more sensitive [29]. Type-specific primers are more sensitive as they amplify short segments of DNA as compared to broad-spectrum or general primers amplifying longer DNA segments [30]. Our data shows that L1 gene detection rates by consensus primers are much lower than E6 and E7 regions of HPV 16 and HPV 18 as detected by type-specific primers. Another possibility is that lesions associated with HPV types 16 and 18 often contain integrated viral DNA at E1/E2 region. This integration also disrupts the normal regulatory function of the E2 gene. It also disrupts the integration of other viral genes including L1 and L2 [31,32]. There is frequent loss of a part of L1 gene when HPV integrates into the host genome at early stages while E6 and E7 are retained [33]. So even if HPV is there, it will not be amplified and will give false-negative results. Other findings have also reported a loss of RNA transcripts of L1/L2 in HPV 16 infected cells [34]. PCRs depending on the presence of these late genes only will underestimate the number of truly HPV positive cases. However, E6 and E7 genes of the oncogenic HPV types being highly conserved in most carcinomas should therefore be detectable in almost all samples in which viral DNA is present [35-37].

Over the last 37 years, the reported frequency of HPV in ESCC varies from 0-100% [17]. A study recently conducted in the high incidence region of MALAWI showed the frequency of HPV to be 15% [38]. HPV has been detected frequently in regions with a high incidence of ESCC i.e. in China 40.1%, South Africa 37.6% and Latin America 22.4%. However, the prevalence of HPV has been found to be low in regions with a low incidence of ESCC such as North America

Table 2. Frequencies of human papillomavirus in ESCC using different primer sets.

Primer set	Tested cases (Number)	Positive cases (Number)	Positive cases (%)
GP+/HPV16+/HPV18+ve *	203	63	31
GP+	203	32	15.7
HPV 16	203	41	20.1
HPV 18	203	16	7.8
Both HPV 16 and HPV 18	203	06	2.9
GP+ and HPV 16/18 -ve **	203	12	5.9

*GP5+/GP6+Positive or HPV 16positive or HPV 18 positive; ** GP5+/GP6+ Positive but HPV 16/18 Negative.

8.0%, Australia 8.1% and Europe 14.0%. This suggests that the etiology of ESCC differs in high and low incidence regions with HPV as an important risk factor only in high incidence regions [12]. Pakistan with a prevalence of 31% can be included in the high incidence regions.

HPV types especially the HR-HPV 16 and 18 are causally linked with squamous cell carcinoma of the cervix, oropharyngeal and esophageal cancers [39-43]. Two types of HR-HPVs identified in our study were HPV 16 and HPV 18 with a prevalence rate of 20.1% and 7.8%. This is in accordance with previous studies indicating HPV type 16 to be the most prevalent type globally in esophageal cancer followed by HPV type 18 as the second most common type [44]. Studies conducted afterward have also reported the same. Yong et al. showed that in esophageal cancer, the prevalence of HPV 16 is 11.7% [30], while Hussain and colleagues [43] found it to be 24.5 %. The results of these studies are comparable to ours. Geographic location seems to be the most important factor affecting the prevalence of HR-HPV in areas with high incidence of ESCC, such as in the regions of Asia. reporting most of the cases [28].

In our study, 6 out of 51 (11.7%) HPV-positive cases by type-specific primers were co-infected similar to as previously reported from some areas of China 11.2% [45]. In a study from Iran, 20.4% of HPV positive samples showed co-infection with HPV types. There were 16 cases positive for HPV 18 but 6 of them showed co-infection with HPV 16, therefore it appears that HPV 18 may be less important as a causative factor of esophageal cancer in this region.

The strength of this study includes a high number of patients as compared to only one study in the same area [46]. Biopsies were taken before any chemotherapy or radiotherapy ensuring no treatment effect. The detection method used was PCR which is considered as highly sensitive [11].

Limitation of the study is the absence of information regarding socioeconomic conditions, tobacco smoking, alcohol consumption, sexual habits and consumption of hot beverages but all these factors have no effects on the PCR results of the study. Formalin fixation affects the quality of DNA extracted, however, the reports are conflicting. Formalin fixation causes DNA degradation as reported by a number of studies, while others suggest no significant effect on DNA amplification [47]. Use of Paraffin embedded formalin fixed tissue is another limitation; however, internal control amplifications were used in every case to minimize these effects. It is also to be noted that ESCC cases in the study were convenient samples and

therefore may not represent ESCC cases in the study area. Although detection of E6/E7 mRNA transcript is regarded as a gold standard for the presence of active (clinically relevant) HPV, it was not done in this study due to the requirement of fresh frozen tissue and additional cost of PCR.

Conclusions

This cross-sectional study showed HPV prevalence of 31% in ESCC. Thus, a subgroup of ESCC may be associated with HPV, however further studies are needed to examine the etiological significance of transcriptionally active HPV by E6/E7 mRNA. Our study also suggests that detection of high-risk HPV types using specific primers for E6 and E7 gene is more sensitive than consensus primers for L1 gene.

Authors' contributions
NS, WS, SP, WI conceptualized the study, participated in its design and coordination. NS, IK, MH, WI, IK, conducted experiments, managed patients, organized and reported data. NS, WS, WI, SP, IK, MH, IK drafted the manuscript and approved the final version.

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