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ORIGINAL ARTICLE

Detection of Merkel Cell Polyomavirus and Human Papillomavirus in Esophageal Squamous Cell Carcinomas and Non-Cancerous Esophageal Samples in Northern Iran

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Abstract Human papillomavirus (HPV) infection is one of the hypothesized causes of esophageal squamous cell carcinoma (ESCC), but the etiological association remains uncertain. It was postulated that other infectious agents together with HPV may increase the risk of ESCC. The current investigation aimed to explore the presence of a new human tumor virus, Merkel cell polyomavirus (MCPyV), together with HPV in ESCC tumors and non-cancerous esophageal samples in northern Iran. In total, 96 esophageal samples (51 with ESCC, and 45 without esophageal malignancy) were examined. HPV DNA was detected in esophageal specimens of 16 out of the 51 ESCC cases (31.4 %) and 20 out of the 45 non-cancerous samples (44.4 %). Untypable HPV genotypes were recognized in high rates in cancerous (75.0 %) and non-cancerous (55.0 %) esophageal specimens. MCPyV DNA was detected in esophageal specimens of 23 out of the 51 ESCC cases (45.1 %) and 16 out of the 45 non-cancerous samples (35.6 %). The mean MCPyV DNA copy number was $1.0 \times 10^{-5} \pm 2.4 \times 10^{-5}$ and $6.0 \times 10^{-6} \pm 1.3 \times 10^{-5}$ per cell in ESCC cases and non-cancerous samples, respectively. There was no statistically significant difference between cancerous and non-cancerous samples regarding mean MCPyV DNA load ($P = 0.353$). A bayesian logistic regression model adjusted to the location of esophageal specimen and MCPyV infection, revealed a significant association between HPV and odds of ESCC (OR, 2.45; 95 % CI: 1.01–6.16). This study provides the evidence of the detection of the MCPyV DNA at a low viral copy number in cancerous and non- cancerous esophageal samples.

Keywords Esophageal squamous cell carcinoma · Merkel cell polyomavirus · Human papillomavirus · Oncogenic viruses

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Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the deadliest malignancies in the world, with a marked geographical variation. Considerable high incidence rates have been identified in a so-called "Asian esophageal cancer belt" stretching from northeast Turkey and Mazandaran in northern Iran through Turkmenistan and central Asian republics to China [1]. Despite decades of epidemiological studies, ESCC accurate etiology remains undetermined. It has been suggested that excessive tobacco smoking, alcohol drinking, exposure to dietary carcinogens and infectious agents may play a role in the pathogenesis of ESCC [2–4]. In terms of infectious etiologies of esophageal cancer, there is accumulating epidemiological evidence suggesting an important role for oncogenic viruses [4]. Among the oncogenic viruses, the presence of human papillomavirus (HPV) in ESCC was demonstrated in numerous studies [5–7]. However, the role of HPV in the etiology of ESCC remains controversial.

Mixed infection with other oncogenic viruses has been postulated to be a cofactor for HPV-related carcinogenesis [8, 9]. Merkel cell polyomavirus (MCPyV) is a new human tumor virus that is convincingly linked to the majority of Merkel cell carcinoma, a rare skin cancer [10]. Merkel cells are neuroepithelial cells that are abundant in the basal layers of the skin, oral mucosa and esophageal epithelium [11, 12]. Recent studies were demonstrated the presence of MCPyV in the upper aerodigestive tract including oral cavity and esophagus [13, 14]. In spite of the presence of susceptible Merkel cells in the esophageal epithelium, infection of esophagus with MCPyV seems probable.

A further rationale for the investigation of MCPyV and HPV in esophageal cancer is waterborne exposure with these viruses. Recently, MCPyV and HPV have been detected in water environments including wastewaters and surface waters [15]. Continuous waterborne exposure of esophageal epithelium with these oncogenic viruses may be correlated with cancer development in this region.

Therefore, the facts reviewed above encouraged us to explore the presence of HPV and MCPyV in the ESCC tumors and non-cancerous esophageal samples in Mazandaran province, which is one of the highest ESCC risk areas in Iran.

Materials and Methods

Clinical Samples

In this cross-sectional study, a total of 96 Formalin-fixed paraffin-embedded resection specimens were collected from the archives of two referral pathology centers in Mazandaran province (Pathology Department of Shahid Beheshti Hospital, affiliated to Babol University of Medical Sciences and Amol Central Pathobiology Laboratory). Out of 96 specimens, 51 had ESCC histopathologic diagnosis and 45 diagnosed without malignant tumor in esophagus. The ESCC tumor samples were categorized based on tumor differentiation grade as follows: of the 51 samples, 33 (64.7 %) were classified as not differentiated tumors, 16 as well and moderately differentiated tumors with 8 samples each (15.7 %) and 2 (3.9 %) samples as poorly differentiated. The distribution of non-cancerous esophageal specimens by histopathologic criteria was as follows: 34 samples (75.6 %) were diagnosed with esophagitis, 6 (13.3 %) samples had esophageal dysplasia and 5 (11.1 %) samples had normal histology. Demographic characteristics (age, gender and residence), information regarding anatomical localization of the esophageal resected specimens (upper third, middle third; and lower third of the esophagus) were collected from patients' medical records (Table 1). None of the participants had received immunosuppressive therapy prior to endoscopy or surgery. This study was approved by the Ethical Committee of Babol University of Medical Sciences, and for all subjects, written informed consent was obtained.

DNA Extraction

Tissue sections (10 μm thick) were deparaffinized according to a previously described procedure [16]. DNA was isolated from each tissue sample, using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. To rule out the possibility of contamination in DNA extraction, in parallel with the tissue samples, negative controls (sterile microcentrifuge tubes containing only reaction mixtures) were included.

HPV Detection and Typing

HPV detection was carried out using the qualitative Real Time PCR with L1 consensus primers (MY09 and MY11) as described previously [6]. Typing was performed in HPV-DNA positive samples using the AmpliSense HPV real-time fluorescence detection (FRT) kit (Central Research Institute of Epidemiology, Moscow, Russia) according to the manufacturer's instructions. This assay can reliably detect 15 different types of HPV, including types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 as high-risk genotypes and types 6 and 11 as low-risk genotypes.

MCPyV Detection and Quantitation

Real-time PCR technique was used to detect and measure the amount of MCPyV DNA load. The viral DNA load was determined as the viral DNA copies per RNase P gene copy (a proven single copy gene), which described the copy number per cell. Preparation of plasmids containing cloned target sequences of MCPyV large T antigen (LT-Ag) and human RNase P gene (real-time PCR standards) was described previously [17, 18]. Quantitative real-time PCR was conducted using an ABI 7300 Real-Time PCR System (Applied Biosystems, Branchburg, NJ, USA) with the primer sets and TaqMan probe specific for the human RNase P gene and MCPyV LT-Ag gene [19, 20]. Total volume of each reaction was 25 μl containing 500 ng of extracted DNA, 12.5 μl YTA 2X Multiplex Real-Time PCR Smart mix (Yekta Tajhiz Azma, Tehran, Iran), 0.3 μl each primer and 0.2 μl dual-labeled probe. To create standard curves, real-time PCR was conducted on a tenfold dilution series of each purified plasmid (pMCPyV LT-Ag and pRNase P) ranging from 2×10^1 to 2×10^6 copies/μl. Each real-time PCR run included reaction mixtures without DNA template as a negative control. To exclude the possibility of contamination and false positive results, a number of control specimens, including whole blood

Table 1 Demographic and clinical characteristics of patients with ESCC tumors and non-cancerous esophageal lesions

Characteristics		ESCCs[a] (n = 51)	Non-cancerous esophageal samples (n = 45)	Total
Gender	Male	30 (58.8 %)	27 (60.0 %)	57 (59.4 %)
	Female	21 (41.2 %)	18 (40.0 %)	39 (40.6 %)
Mean age (yr)		69.1 ± 11.1	65.4 ± 12.4	67.4 ± 11.8
Residence	Urban	26 (51.0 %)	25 (55.6 %)	51 (53.1 %)
	Rural	25 (49.0 %)	20 (44.4 %)	45 (46.9 %)
Location of esophageal specimen	Upper third	16 (31.4 %)	6 (13.3 %)	22 (22.9 %)
	Middle third	20 (39.2 %)	6 (13.3 %)	26 (27.1 %)
	Lower third	15 (29.4 %)	24 (53.3 %)	39 (40.6 %)
	Unknown	0 (0 %)	9 (20 %)	9 (9.4 %)

a. Esophageal squamous cell carcinomas

and serum from healthy subjects, in which the presence of MCPyV was not suspected, were analyzed.

Statistical Analysis

Statistical analyses were performed using arm and base packages of R software, version 3.0.0 [21]. Normal distribution of the quantitative variables was checked by Kolmogorov–Smirnov and Shapiro–Wilk tests. The differences between normal variables were analyzed by t test or analysis of variance. Multiple comparisons were performed using Tukey test. The differences between nonparametric variables were compared by Mann–Whitney U test or Kruskal–Wallis test. According to the small number of samples, Bayesian logistic regression was used to calculate odds ratios (ORs) and 95 % confidence intervals. P-value of ≤0.05 was considered to be statistically significant.

Results

In the present cross-sectional study, a total of 96 esophageal specimens were investigated. Study subjects were divided in two groups: patients with diagnosed ESCC (n = 51) and those without diagnosed malignant tumor in esophagus (n = 45). The mean age of the subjects in ESCC group (male 30, female 21) was 69.1 ± 11.1 years (range 38–91), whereas the mean age of the individuals in non-cancerous group (male 27, female 18) was 65.4 ± 12.4 years (range 42–90). There was no statistically significant difference between age of the groups (P = 0.127). In terms of urban/rural residence, from the 51 patients in ESCC group 26 (51 %) were urban and 25 (49 %) were rural. Of 45 patients in non-cancerous group, 25 (55.6 %) and 20 (44.4 %) lived in urban and rural areas respectively. Considering location of esophageal specimens, in ESCC group the most common site was middle third of esophagus with 20 cases (39.2 %), followed by upper third and lower third with 16 (31.4 %) and 15 (29.4 %) cases respectively. Of the 45 non-cancerous esophageal specimens, 24 cases (53.3 %) were located at lower third of esophagus and 12 cases were located at upper and middle third, with 6 cases each (13.3 %). The location of specimen was unknown in 9 (20 %) subjects in non-cancerous group.

The results from HPV detection and genotyping revealed the presence of HPV DNA sequences in a total of 36 (37.5 %) out of the 96 tested samples. HPV DNA was detected in esophageal specimens of 16 out of the 51 ESCC cases (31.4 %) and 20 out of the 45 non-cancerous group (44.4 %). In ESCC group, 13.7 % (7/51) of not differentiated and 2.0 % (1/51) of well differentiated tumors was only HPV DNA positive. Single HPV infection was not detected in moderately and poorly differentiated tumors. In non-cancerous group, single HPV infection was detected in 20 % (9/45) of esophagitis, 4.4 % (2/45) of esophageal dysplasia, and 2.2 % (1/45) of samples with normal histology (Table 2). Among ESCC cases, 3 out of 16 samples (18.7 %) were infected with high-risk types, compared to 5 out of the 20 samples (25 %) in non-cancerous group. Infections with multiple HPV types were seen in samples from 3 ESCC cases, but in none of the 20 samples from non-cancerous group. Out of 16 HPV-positive samples in ESCC group, 12 (75.0 %) were untypable, one sample was infected with HPV11, two samples were multiple-infected with two genotypes (HPV16/HPV45 and HPV35/HPV52), and one sample was multiple-infected with three genotypes (HPV39/HPV45/HPV59). Of the 20 HPV positive non-cancerous esophageal specimens, 11 (55.0 %) were untypable, 4 (20 %) samples were infected with HPV11, and each of the following genotypes, including HPV33, HPV39, HPV52, HPV56 and HPV58 were detected in one sample (5 %).

In the current study, cancerous and non-cancerous esophageal specimens were tested for the presence of MCPyV LT-Ag sequence by quantitative real-time PCR. Of the 96 tested samples, the MCPyV LT-Ag sequence was found in 39 (40.6 %). MCPyV DNA was detected in esophageal specimens of 23 out of the 51 ESCC cases (45.1 %) and 16 out of the 45 non-cancerous group (35.6 %). Regarding the grade of tumor in ESCC group, single MCPyV infection was detected in 17.6 %

Table 2 Frequency of HPV and MCPyV infection in ESCC cases and non-cancerous esophageal specimens according to the histopathologic diagnosis

Histopathologic diagnosis in study groups		Total	Single HPV infection (%)	Single MCPyV infection (%)	HPV and MCPyV coinfection (%)	*P*-value
ESCC group (*N* = 51)	Well differentiated ESCC[a]	8 (15.7)	1 (2.0)	3 (5.9)	1 (2.0)	0.903
	Moderately differentiated ESCC	8 (15.7)	0 (0.0)	2 (3.9)	1 (2.0)	
	Poorly Differentiated ESCC	2 (3.9)	0 (0.0)	1 (2.0)	0 (0.0)	
	Not differentiated ESCC	33 (64.7)	7 (13.7)	9 (17.6)	6 (11.8)	
Non-cancerous group (*N* = 45)	Dysplasia	6 (13.3)	2(4.4)	2 (4.4)	1 (2.2)	0.444
	Esophagitis	34 (75.6)	9 (20.0)	6 (13.3)	7 (15.6)	
	Normal	5 (11.1)	1 (2.2)	0 (0.0)	0 (0.0)	

[a] Esophageal squamous cell carcinomas

(9/51) of not differentiated, 2.0 % (1/51) of poorly differentiated, 3.9 % (2/51) of moderately differentiated, and 5.9 % (3/51) of well differentiated tumors. In non-cancerous group, 13.3 % (6/45) of esophagitis, and 4.4 % (2/45) of esophageal dysplasia specimens contained only MCPyV LT-Ag sequence. Merkel cell polyomavirus LT-Ag sequence was not found in samples with normal histology. Concomitant infection with HPV and MCPyV was seen in 8 ESCC cases (15.8 %) and 8 non-cancerous esophageal specimens (17.8 %). With respect to the grade of tumor in ESCC group, HPV and MCPyV concomitant infection was detected in 11.8 % (6/51) of not differentiated, 2.0 % (1/51) of moderately differentiated, and 2.0 % (1/51) of well differentiated tumors. In non-cancerous group, 15.6 % (7/45) of esophagitis, and 2.2 % (1/45) of esophageal dysplasia showed HPV and MCPyV concomitant infection (Table 2).

The MCPyV LT-Ag DNA load was determined as the viral DNA copies per RNase P gene copy (a proven single copy gene), which described the viral copy number per cell. The mean MCPyV LT-Ag copy number was $1.0 \times 10^{-5} \pm 2.4 \times 10^{-5}$ and $6.0 \times 10^{-6} \pm 1.3 \times 10^{-5}$ per cell in ESCC cases and non-cancerous samples, respectively. There was no statistically significant difference between ESCC cases and non-cancerous samples regarding mean MCPyV LT-Ag DNA load ($P = 0.353$). Among MCPyV–positive ESCC tumors, the mean DNA load was significantly higher in poorly differentiated ESCCs ($4.1 \times 10^{-5} \pm 5.7 \times 10^{-5}$copies per cell) compared with moderately ($3.0 \times 10^{-6} \pm 7.0 \times 10^{-6}$copies per cell) and well differentiated ($2.6 \times 10^{-5} \pm 4.8 \times 10^{-5}$copies per cell) tumors ($P = 0.043$) (Fig. 1). In addition, MCPyV LT-Ag copy number was significantly higher in well differentiated ($2.6 \times 10^{-5} \pm 4.8 \times 10^{-5}$copies per cell) ESCCs in comparison with not differentiated ($6.0 \times 10^{-6} \pm 1.2 \times 10^{-5}$copies per cell) samples ($P = 0.034$) (Fig. 1). In MCPyV-positive non- cancerous samples, the mean MCPyV DNA copy number was higher in samples with esophagitis ($7.0 \times 10^{-6} \pm 1.5 \times 10^{-5}$copies per cell) compared to esophageal dysplasia ($3.0 \times 10^{-6} \pm 3.0 \times 10^{-6}$copies per cell); but this difference was not statistically significant ($P = 0.229$).

To evaluate HPV and MCPyV interaction effect in ESCC development, a bayesian logistic regression model was applied. According to aforementioned model there was no statistically significant interaction between HPV and MCPyV in ESCC development (OR, 0.94; 95 % CI: 0.17–5.19). However, when the model was adjusted for the location of esophageal specimen and MCPyV infection, a significant association between HPV and odds of ESCC was observed (OR, 2.45; 95 % CI: 1.01–6.16) (Table 3). In addition, adjustment of aforementioned model for HPV and MCPyV infection decrease the odds of ESCC in lower third of esophagus (OR, 0.29; 95 % CI: 0.09–0.85) (Table 3).

Discussion

The etiology of esophageal cancer has not been well-understood, and the risk factors between Western and Asian countries appear different [22]. In northern Iran, ESCC is the dominant type of esophageal malignancy with markedly high incidence rates [23]. Implication of HPV as a contributor to ESCC has been proposed by several investigations [5, 7, 24]. However, there is no consensus on the role of HPV in esophageal carcinogenesis. It was suggested that other infections together with HPV may increase the risk of ESCC [4]. The role of infection with MCPyV as a human tumor virus in the etiology of ESCC is not clear.

In the present cross-sectional study, a total of 96 cancerous and non-cancerous esophageal samples were examined for HPV

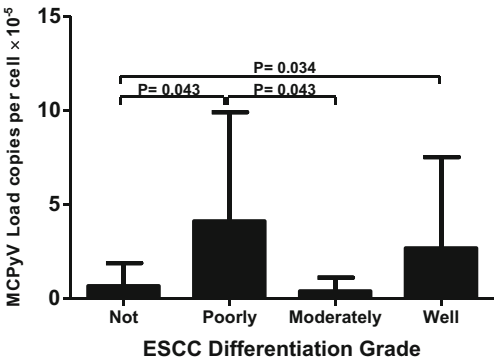

Fig. 1 The mean MCPyV LT-Ag DNA load in MCPyV positive ESCC tumors according to tumor differentiation grade. Error bars indicate standard error. The *P*-value was determined by Tukey multiple comparison test

Table 3 Bayesian logistic regression analysis included location of esophageal specimen, HPV and MCPyV infection

Parameter		Odds Ratio (OR) of ESCC[a]	95 % Confidence interval		*P*-value
			Lower	Upper	
MCPyV infection		0.59	0.23	1.49	0.26
HPV infection		2.45	1.01	6.16	0.49
Location of esophageal specimen*	Middle third	1.39	0.41	4.75	0.59
	Lower third	0.29	0.09	0.85	0.02

*Upper third of esophagus is taken as a reference category

[a] Esophageal squamous cell carcinoma

and MCPyV infections. HPV DNA was detected in 31.4 % of samples with ESCC, and in 44.4 % of non-cancerous samples. The higher rate of HPV infection in non-cancerous esophageal samples might be explained by the source of samples in the current study (mostly esophagitis and esophageal dysplasia) compared to some previously published studies (mostly normal esophageal mucosa) [25]. In total, 10 different HPV types, including 9 high-risk types and 1 low-risk type were identified. Infection with HPV16 was detected in very low rate; only 1 ESCC sample with HPV16 genotype was recognized. The results of HPV genotyping in the present investigation are inconsistent with a number of reports, which identified HPV16 as a most prevalent type in ESCC [26]. However, HPV infections caused by unknown or untypable genotypes were recognized in high rates in ESCC cases (75.0 %) and non-cancerous group (55.0 %). Untypable genotypes represent HPV types other than those identified by the genotyping system, or a low viral gene copy number, which thus escape genotype recognition [27]. Although the tumorigenic role of untypable HPV infection seems limited, there is little data on the relationship between these types of infection and premalignant transformation of squamous cells on the surface of the cervix [28].

In the present study, different grades of ESCC tumors, esophagitis, esophageal dysplasia and normal esophageal samples were analyzed for the MCPyV LT-Ag oncogene sequence. Merkel cell polyomavirus LT-Ag sequence was found in 45.1 % of ESCC tumor specimens and 35.6 % of non-cancerous esophageal samples. Viral oncogene sequence was detected in all differentiation grades of ESCC tumors. In addition, MCPyV infection was found in esophagitis and esophageal dysplasia specimens. However, none of the esophageal samples with normal histology were MCPyV DNA positive. To the best of our knowledge, there is only one published pilot study with limited sample size, which has indicated the presence of MCPyV sequences in normal esophagus and esophageal cancer [14].

In the current study, a low copy number of MCPyV LT-Ag gene per cell was detected in cancerous and non-cancerous esophageal samples. Normalization of viral gene copy numbers to cell numbers is more valuable when evaluating viral loads in clinical samples [29]. The existence of low copy numbers of MCPyV genome in tumors is a matter for debate. The Merkel cell polyomavirus genome is present generally at more than one DNA copy per Merkel cell carcinoma tumor cell, supporting a direct tumorigenic mechanism and tumor-viral clonality [30]. The existence of less than one MCPyV DNA copy per cell in ESCC and non-cancerous esophageal samples might be explained by a few possibilities. The first explanation could be the shed of virus from another organ (e.g. respiratory tract) without any pathological consequence. Recent evidence has demonstrated the presence of MCPyV in respiratory tract and respiratory secretions [34]. Second, low copy numbers of MCPyV genome in esophageal tissue might be explained by simple persistent viral replication without any role in tumor induction. In addition, a number of studies were demonstrated that the expression of the MCPyV LT-Ag is pivotal for the viral oncogenesis [31–33]. Due to our current sample set (Formalin-fixed paraffin-embedded samples) we were unable to examine viral LT-Ag expression. In the current investigation, MCPyV LT-Ag sequence was not found in samples with normal histology. In addition HPV DNA was quantified in only one esophageal normal sample. This finding might be cautiously relevant to the role of these viruses in esophageal oncogenesis, but further studies with higher sample size and matching normal controls should be done to clear the role of aforementioned viruses in esophageal oncogenesis.

According to bayesian logistic regression model, a positive association between HPV infection and ESCC development was seen, after adjusting for location of esophageal specimen and MCPyV infection, which means, by controlling the effect of location of specimen and MCPyV infection, HPV has the highest effect on the ESCC development. Also, controlling the effect of HPV and MCPyV variables, decrease the odds of ESCC in lower third of esophagus.

In conclusion, the present study provides the evidence of the detection of the MCPyV LT-Ag sequence at a low viral copy number in cancerous and non- cancerous esophageal samples. This study suggests that MCPyV can infect esophageal tissues, either alone or together with HPV. It is clear that the mere detection of HPV or MCPyV DNA sequences is not enough for etiologic contribution in malignant transformation. Further epidemiological and molecular studies, especially on esophageal fresh biopsy samples and in case-control setting

should be done to understand the role of HPV and MCPyV in esophageal oncogenic transformation.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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