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RESEARCH

High-Risk and Low-Risk Human Papillomavirus in Esophageal Squamous Cell Carcinoma at Mazandaran, Northern Iran

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Abstract Cancers are the second most common cause of non-accidental deaths in Iran, following cardiovascular deaths. Mazandaran, near the Caspian Littoral at north of Iran have identified as a several-high incidence area for Esophageal Squamous Cell Carcinoma (ESCC) in the world. Several associated risk factors, such as dietary and cultural habits, infectious agents, nutritional deficiencies, too much use of tobacco and alcohol and infection to certain DNA tumor viruses (HPVs), including environmental and genetic factors are attributed to this disease. To explore this issue, we analyzed HPV DNA prevalence and HPV types together in relation to tumor sites a high-incidence population. Archived tissue blocks from 46, 69 and 62 upper, middle and lower third of esophagus, respectively from ESCC patients were evaluated for the presence of HPV DNA by PCR using the degenerate HPV L1 consensus primer pairs MY09/MY11. The positive specimens were evaluated by Real-time PCR to determine HPV genotypes. From the 49 HPV positive cases, of ESCC patients, 5 (23.1 %), 11 (55 %) and 9 (56.3 %) of upper, middle and lower third of ESCC specimens, respectively were positive by at least one high and one low-risk HPV genotypes. In general, HPV45 and HPV11 were the most common high- risk and low-risk HPV genotypes in HPV L1 positive cases, respectively, followed by HPV6, HPV52 and HPV39. Therefore, the high prevalence of HPV DNA in different anatomical sites of ESCC patients from the Mazandaran region in North of Iran provides more evidence for a role of HPV in this cancer.

Keywords High-risk and Low-risk HPV · Genotyping · ESCC · Mazandaran · North of Iran

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Introduction

Cancers are among the most common causes of death throughout the world. It is estimated that the overall incidence of various types of cancers will increase by 45 % in developed countries by 2030. Recent reports indicate that cancers are the second most common cause of non-accidental deaths in Iran, following cardiovascular deaths [1–3].

Epidemiological studies have identified several-high incidence area in China, Singapore, Iran, Russia, Puerto Rico, Chile, Brazil, Switzerland, France and South Africa [4, 5], but the causes for striking geographical variations in the incidence of human esophageal cancer remain ambiguous. In India, esophageal cancer is most common, but unevenly distributed with certain regions showing a higher prevalence, and, recently, an increasing trend in the rate of its incidence has been observed [6]. Several associated risk factors, such as dietary and cultural habits, infectious agents, nutritional deficiencies, too much use of tobacco and

alcohol and infection to certain DNA tumor viruses (HPVs, EBV), including environmental and genetic factors are attributed to this disease [7–10].

In the USA and other western countries tobacco, too much alcohol drinking, diets poor in fresh fruits and vegetables, and low socioeconomic group have been associated with SCC [11, 12], but a potential role of HPV has been proposed [13, 14]. In Iran and China, alcohol and tobacco are not considered as risk factors [15–20].

To date, more than 140 HPV genotypes have been recognized and subdivided into cutaneous and mucosal HPV types. HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82 are classified as high-risk (HR) types; types 6, 11, 40, 42, 44, 54, 61, 70, 72, 81, and CP6108 are classified as low-risk (LR) types; and types 26, 53, and 66 are considered as probably oncogenic [21, 22]. Also, HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 66 are carcinogenic to humans (Group 1). HPV 6 and HPV 11 (Group 2B) and some types of HPV genus beta are possibly carcinogenic to human (Group 2B).

Interestingly, studies from Asian countries, especially China, have reported relatively high percentages of HPV positive ESCC cases when compared to reports from Western European countries. There are areas, particularly in Asia, where HPV is more commonly detected in esophageal cancer. Recent case series have been reported from Egypt [23], Colombia and Chile [24], Brazil [25], Germany [26], the Republic of Korea [27], the Islamic Republic of Iran [28], and China [14, 29]. HPV detection in these recent studies ranged from 0 % (Republic of Korea) to 54 % (Egypt). HPV 16 was the most common type in all studies, followed by HPV 18.

To our knowledge, there is no study comparing the HPV infection rates in the North of Iran. In the present study, we examined ESCC in Mazandaran province, Northern Iran detecting genotypes of high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59) and low-risk HPV types (6, 11) with respect to different parts of esophagus.

Materials and Methods

Patients

We examined 177 formalin-fixed and paraffin-embedded ESCC specimens (46, 69 and 62 cases of upper, middle and lower third, respectively) from Shahid Beheshti Hospital, Babol and Amol Central Laboratory in Mazandaran, Northern Iran, from 2004 to 2011. The patient habitats in about 15 cities of Mazandaran province, as a region in high incidence of esophageal cancer. In every case, the pathological diagnoses were rendered by local pathologists. The demographic and medical information including age, gender, habitant, tumor site was obtained from patient medical records.

DNA Extraction

Five to 10 slides (depends on type of the specimen: biopsy or surgery) were deparaffinized in xylene and absolute alcohol, then the lysis buffer (300 mmol/l NaCl; 50 mmol/l Tris·HCl pH 8.0; 0.2 % SDS) was added into the tube with proteinase K (200 mg/l), and the solution was incubated at 55 °C overnight until it became clear. Then DNA was extracted using High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim Germany).

Real-Time PCR for Qualitative Detection of HPV-DNA

All DNA extracted from ESCC specimens and positive or negative controls for HPV detection and genotyping performed with high-sensitive real-time PCR (Corbett Rotor-Gene 6000 Sequence Detection System). The 12-μl reaction mixture included and SYBR-Green PCR master mix (Maxima® SYBR Green qPCR Master Mix (2X), Applied Fermentas, EU); forward and reverse primers at 10 pmol per reaction for MY09 (5′-CGT CCM AAR GGA WAC TGA TC-3′) and MY11 (5′-GCM CAG GGW CAT AAY AAT GG-3′) from L1 genes; and 6 μl DNA sample. PCR reactions were as follows: The amplification conditions were a three-step cycle of 95 °C for 10 min (for initial denaturation), 95 °C for 15 s, 55 °C for 30s and 72 °C for 30s (5 repeats). For cycling 2: 95 °C for 15 s, 55 °C for 30s and 72 °C for 30s for a total of 40 cycles.

Real-Time PCR for HR&LR-HPV

All HPV-DNA positive specimens of ESCC for HPV genotyping performed with high-sensitive real-time PCR (Rotor Gene 6000, Corbett Research, Australia & USA). The 13-μl reaction mixture included AmpliSense HPV HCR genotype FRT PCR Kit for High-Risk genotyping and then all HPV-DNA positive samples were tested by AmpliSense HPV 6/11 FRT PCR Kit for detection of Low-risk genotyping (Federal State Institution of Science Central Research Institute of Epidemiology, 3A Novogireevskaya Street Moscow 111123 Russia).

Quality Control

DNA preparation, PCR setup, and PCR product detection were approved in separated spaces with specimens moving through the laboratory in one direction only. The

human β-globin gene and HPV L1 were tested in these controls to confirm the absence of contamination during DNA preparation.

In each PCR reaction tube the following controls were included: β-globin as a Housekeeping gene for DNA preparation control (β-globin Forward: 5′-TGG GTT TCT GAT AGG CAC TGA CT-3′; β-globin Reverse: 5′-AAC AGC ATC AGG AGT GGA CAG AT-3′); negative controls without a DNA template; and one positive control containing HPV-18 was extracted on Hela cell line and HPV-16 and HPV-18 approved by WHO.

Assay results were interpreted and used for this study only when controls met the following criteria: 1. All negative controls were negative; 2. ESCC DNA was positive; and 3. Positive control was positive. In cases where any negative control was positive, testing was repeated.

Statistical Analysis

A $\chi 2$ test was used to compare categorical data. A P-value less than 0.05 were considered statistically significant. Statistical analysis was performed with SPSS 14.

Results

The mean ± SD age studied patients for the 46 upper, 69 middle and 62 lower third of esophagus was 67.6±9.7, 65.6 ±11.0 and 67.4±11.9 years, respectively. 58.7 %, 56.9 % and 50 % of upper, middle and lower specimens belong to males. We found no difference with respect to age ($\chi 2$= 4.03; df=6; ρ=0.67), gender ($\chi 2$=0.94; df=2; ρ=0.63) and anatomical sites of esophagus (as shown in Table 1).

From 46 (28.3 %) upper, 69 (29 %) middle and 62 (25.8 %) lower third of ESCC cases 28.3 %, 29 % and 25.8 % were HPV L1 positive, respectively (Fig. 1) ($\chi 2$= 0.175; df=2; ρ=0.916). Using the Real-time PCR method, a total of 14 genotypes including HPV6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59 were detected. Of 49 HPV L1 positive specimens of ESCC, 23.1 % of patient in upper, 55 % in middle and 56.3 % of them in lower third of ESCC samples were positive by at least one of the high and low-risk HPV genotypes. The most prevalent genotype was HPV11 and HPV45 was detected in 20 % in middle third of the HPV L1 positive cases. HPV6 was the second most prevalent genotype in middle third and was found in 15 %, followed by HPV39 in 10 % of HPV L1 positive cases of middle third of ESCC specimens. HPV52 was the more common prevalent genotype (18.8 %) in lower third and the second more prevalent genotypes in this site of esophagus were HPV11, 39 and 45 with 12.5 % for each type. In addition, many other HPV genotypes were found, although less common, in every three third of ESCC cases (Table 2 and Fig. 2). Also, among HPV L1 positive cases, 26.5 % were infected by single genotype and 20.4 % of them were co-infected with more than one HPV type (Table 3).

As shown in Table 1 the difference between the numbers of HPV genotypes according to each part of esophagus are statistically significant.

Discussion

In the present series, men and women were nearly equal represented, a gender distribution similar to most high incidence areas where tobacco and alcohol are not the main risk factors for ESCC, such as in northeastern or in central China [30–32]. Molecular evidence of ESCC in Mazandaran appears different from those in high incidence areas of HPV infection.

To our knowledge, this study is the first report on the molecular evaluated of ESCC from patients of Mazandaran province, a possibly high incidence area in northern Iran.

Table 1 The description of gender, age and HPV positivity by different parts of esophagus in ESCC patients

	Number of patients (N/%)	Upper third (N/%)	Middle third (N/%)	Lower third (N/%)
Gender				
Male	98 (55.4)	27 (58.7)	39 (56.5)	31 (50)
Female	79 (44.6)	19 (41.3)	30 (43.5)	31 (50)
Age				
<55	35 (19.8)	6 (13)	17 (24.6)	12 (19.4)
55 to 64	28 (15.8)	10 (21.7)	9 (13)	9 (14.5)
65 to 74	69 (39)	20 (43.5)	25 (36.2)	24 (38.7)
>=75	45 (25.4)	10 (21.7)	18 (26.1)	17 (27.4)
HPV infection				
Positive	49 (27.7)	13 (28.3)	20 (29)	16 (25.8)
Negative	128 (72.3)	33 (71.2)	49 (71)	46 (74.2)

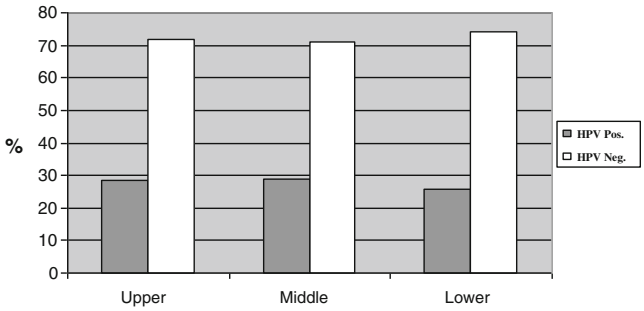

Fig. 1 Percentage of HPV L1 DNA in different part of esophagus in ESCC patients

Although limited to 177 cases, this series is of interest since there is no report for ESCC in Iran except for a study in Tehran, Central of Iran [28] and Turkmen Sahra in Northeast Iran [33].

We observed a prevalence of HPV DNA of 27.7 %, far below the high prevalence reported in high incidence areas of China (30–82.8 %) [14, 29, 34–36], Northeast Iran (49 %) [33], or South African (44–46 %) [37, 38]. The earlier studies in ESCC samples of Iranian patients that associated HPV with 23.6 % [28] and 36.8 % [39] in Central of Iran, and 49.4 % [33] of cases of esophageal cancer in northeast of Iran used PCR.

We employed a sensitive real-time PCR assay for analysis of paraffin-embedded tissue from patients with Esophageal Squamous Cell Carcinoma According to Fig. 2 many HPV genotypes have not been previously reported in other ESCC studies of Iran.

Table 2 Detection of HPV genotypes in HPV L1 positive in different anatomical sites of patients with ESCC

HPV genotype	Third of esophagus		
	Upper	Middle	Lower
HPV-6	1	3	1
HPV-11	0	5	2
HPV-16	0	0	1
HPV-18	0	1	0
HPV-31	1	1	0
HPV-33	1	1	0
HPV-35	0	1	1
HPV-39	0	2	2
HPV-45	1	4	2
HPV-51	0	0	0
HPV-52	1	1	3
HPV-56	0	1	1
HPV-58	0	0	1
HPV-59	0	1	0
Others (Unknown)	10	9	7

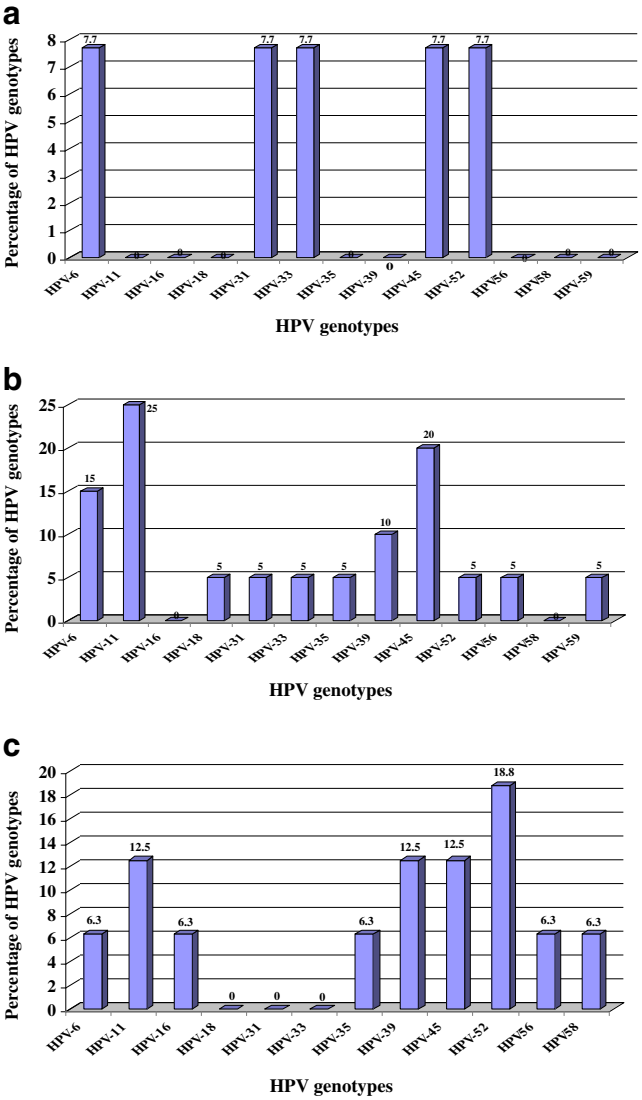

Fig. 2 Relative frequency (%) of HPV genotypes in HPV L1 positive patients with ESCC in Mazandaran, near the Caspian Littoral. **a** Upper; **b** Middle and (**c**) Lower third of Esophagus

We also detected HPV genotypes 39, 45, 56, 58 and 59 which has never before been shown before present in ESCC in Iran and many other countries. In contrast, the earlier studies have shown that HPV types 16 and 18 are more commonly detected and more frequently associated with ESCC. Also, HPV genotype 11 and 6 are two of the predominant type in this study. In our study, we found HPV type 16 DNA and HPV type 18 DNA were present in only one of the lower (2 %) and middle part (2 %) of HPV positive cases.

In general, HPV type 52 DNA was present in 10.2 % and each one of HPV types 31, 33, 35 and 56 DNA was present in 4.1 % in HPV DNA positive cases. Also, HPV types 58 and 59 DNA was present in only one sample whereas, HPV type 11 was the most predominant (22.4 %) (Table 2). The

Table 3 Samples of Patients infected with Single and multiple HPV infection

No.	Sample code	HPV genotypes
1	10	39, 45, 59
2	29	11
3	57	6
4	64	6
5	65	11
6	72	52
7	74	16, 45
8	82	11
9	91	39
10	101	11
11	118	6
12	146	11, 45
13	149	11, 31, 33,35, 56
14	152	39, 45
15	161	6, 39, 45
16	163	11, 45, 52
17	165	35, 52
18	171	31, 33, 52
19	172	6
20	185	52, 58
21	187	45
22	188	18
23	203	56

earlier studies in Iran showed, HPV type 16, 18, 31 and 33 [28], only HPV type DNA 16 and 18 [39] and also HPV type DNA 16, 18, 6, 66 and 52 [33].

In European study in Uppsala, Sweden, 16 % of patient with ESCC have HPV DNA by real-time PCR and they reported only HPV genotype 16 DNA between HPV types of 16, 18, 31, 33, 35, 45, 52 and 67 [40].

In Transkei of South Africa, researchers found HPV type 16 DNA was present in only two cases, whereas HPV type 18 DNA was not detected. Also, they reported that HPV type 52 DNA was present in only one case, whereas similar to our study, HPV type 11 was the most predominant type [37]. We also identified HPV type 39, which has never been shown before to be present in esophageal cancer in our country and many other countries but this type has been reported by Matsha et al. [37]. In a report in Latin-American region, they found HPV in 25 % of ESCCs and HPV-16 was the most frequent observed genotype, followed by both HPV-18 and HPV-59 in one case [41].

The detection of HPV 16 in malignant cases is more frequently compared to HPV 18 [25, 26, 34, 41] or other types including HPV 33 and 31 [28] and 'low-risk' HPVs including HPV 11 [42]. In the study by Matsha et al. [38], similar to our study, they showed that HPV 11 and 39 were detected more frequently than HPV 16, suggesting a possible role of HPV types other than 16 and 18 in the pathogenesis of esophageal cancer.

Among the three areas of China study, HPV6, 16, 18, 26, 45, 56, 57, and 58 were identified in positive specimens by sequencing, but HPV16 and 57 were the most common types in all regions, followed by HPV26 and HPV18. From the HPV types reported in human ESCC from Colombia and Chile, HPV-16 has been the most commonly identified, followed by other high-risk HPV types, including HPV-18, HPV-31, and HPV-35 [24]. HPV DNA were detected in 26.7 % of Indian patients with ESCC whiles HPV-16 and 18 were detected in 18.8 % of cases [43].

The 13 types of HPV identified in our study that 11 types of them (HPVs-16, 18, 31, 33, 35, 39, 45, 52, 58 and 59) are high-risk HPV types (potentially cancer causing; ref. 21 and 22), and HPV-6 and HPV-11 being the most commonly detected low-risk HPV type. In our study, many specimens had multiple HPV infections (Table 3). Among HPV positive specimens, 20.4 % were co-infected with more than one HPV type that was comparable to 15.8 % cases from China [34]. This seems different from most studies, although double or even multiple HPV infections have been reported in ESCC from some areas of China [29].

The HPV transmission route in ESCCs is also of interest. HPV infection of the esophageal mucosa is highly suspected to occur in a direct route. In a recent case–control study [43], HPV oral infection was strongly associated with a sub-group of oropharyngeal squamous cell carcinomas, in which high-risk sexual behaviors (i.e. oral, vaginal) were recorded, regardless of alcohol and tobacco use. In spite of the association between oral HPV infection and sexual behavior, a Finnish HPV Family Study [44] has shown that persistent high-risk HPV infection in a mother is a major risk factor for oral and genital infections by this virus in her children; this susceptibility appears to be modulated by the immune system. Thus, it could be argued that previous high-risk HPV oral infection might predispose asymptomatic carriers for further ESCC progress.

Conclusion

To our knowledge, this is the first survey evaluating the prevalence of HPV infection in Mazandaran, and drawing attention to the unusual high proportion of many genotypes of HPV except HPV16 and 18. We found evidence of an association between esophageal squamous cell carcinoma and HPV in the population studied. However, the findings here serve to raise the possibility that HPV plays a role in the etiology of esophageal carcinoma.

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