



Human papillomavirus in upper digestive tract tumors from three countries

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Abstract

AIM: To clarify human papillomavirus (HPV) involvement in carcinogenesis of the upper digestive tract of virological and pathological analyses.

METHODS: The present study examined the presence of HPV in squamous cell carcinomas of the oral cavity ($n = 71$), and esophagus ($n = 166$) collected from Japan, Pakistan and Colombia, with different HPV exposure risk and genetic backgrounds. The viral load and physical status of HPV16 and HPV16-E6 variants were examined. Comparison of *p53* and *p16^{INK4a}* expression in HPV-positive and HPV-negative cases was also made.

RESULTS: HPV16 was found in 39 (55%) oral carcinomas (OCs) and 24 (14%) esophageal carcinomas (ECs). This site-specific difference in HPV detection between OCs and ECs was statistically significant ($P < 0.001$). There was a significant difference in the geographical distribution of HPV16-E6 variants. Multiple infections of different HPV types were found in 13 ECs, but multiple infections were not found in OCs. This difference was statistically significant ($P = 0.001$). The geometric means (95% confidence interval) of HPV16 viral load in OCs and ECs were 0.06 (0.02-0.18) and 0.12 (0.05-0.27) copies per cell, respectively. The expression of *p16^{INK4a}* proteins was increased by the presence of HPV in ECs (53% and 33% in HPV-positive and -negative ECs, respectively; $P = 0.036$), and the high-risk type of the HPV genome was not detected in surrounding normal esophageal mucosa of HPV-positive ECs.

CONCLUSION: Based on our results, we cannot deny the possibility of HPV16 involvement in the carcinogenesis of the esophagus.

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Key words: Human papillomavirus; Viral load; Physical status; E6; p53; p16^{INK4a}

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INTRODUCTION

Human papillomaviruses (HPVs) belong to the *Papillomaviridae* family and are non-enveloped icosahedral viruses with a diameter of 55 nm and have more than 100 types^[1]. The International Agency for Research on Cancer considers that there is convincing evidence indicating that infection with *HPV16*, *18*, *31*, *33*, *35*, *39*, *45*, *51*, *52*, *56*, *58*, *59* or *66* can lead to cervical cancer^[2]. To date, approximately 20 types have been identified as high-risk HPVs that increase the risk of cervical cancer. Among them, *HPV16* and *HPV18* are considered to be associated with 70% of all cervical cancer cases^[2]. In contrast, low-risk HPV types such as *HPV6* and *HPV11* cause genital warts but not cancer.

The association of HPV with cancers of the upper digestive tract (UDT) is also suspected. Major malignancies observed in the UDT include cancers of the oral cavity, oropharynx, larynx and esophagus. Meta-analysis of 4680 samples from 94 reports published between 1982 and 1997 has shown that HPV was 2-3 times more likely to be detected in precancerous oral mucosa and approximately five times more likely to be detected in oral carcinoma (OC) than in normal mucosa^[3]. Among the studies used in this meta-analysis, the largest and best-designed study was that by Maden *et al*^[4]. They examined 112 normal mucosal specimens and 118 OCs and detected *HPV16* in six (5%) cases of OC but in only one (0.9%) normal mucosal specimen. In contrast, *HPV6* was detected in 12 OCs and 10 normal mucosal specimens. High-risk HPV has also been detected in esophageal carcinomas (ECs). A review of studies published between 1982 and 2001 has shown that 15.2% of the 2020 squamous cell carcinomas (SCCs) of the esophagus tested using polymerase chain reaction (PCR) were *HPV* positive^[5]. However, previous studies have shown various *HPV*-positive rates in non-genital cancers worldwide. One argument is that this difference was caused by different *HPV*-detection methods with different sensitivity and specificity among studies. Another possible explanation is different *HPV* exposure risk and/

or susceptibility of disease/infection across study populations.

Furthermore, the role of *HPV* in UDT carcinomas, particularly ECs, remains unclear and controversial^[6]. Two European prospective serological studies that used stored serum specimens^[7,8] and a Chinese case-control study^[9] have found a strong association between the risk of ECs and seropositivity for *HPV16*. In contrast, two retrospective studies conducted in Europe^[10,11] and a large prospective serological study in China^[12] have found no significant association of *HPV16* or *HPV18* with ECs.

In the present study, cases of oral cavity and esophageal cancer were examined for concomitant HPV infection, the type of HPV involved, and multiple infection with different types of *HPV* in Japan, Pakistan and Colombia, with different *HPV* exposure risks and genetic backgrounds, using the same methods. In order to shed light on the etiological significance of *HPV* in the development of OCs and ECs, the viral load and physical status of *HPV16* (which is the most commonly found HPV type worldwide) and *HPV16*-E6 variants were examined. Comparison of *p53* and *p16*^{INK4a} expression in HPV-positive and HPV-negative OCs and ECs was also made.

MATERIALS AND METHODS

Ethics

Institutional Review Board of the Faculty of Medicine, Kagoshima University, Japan, approved the present study.

Subjects

This study examined 261 formalin-fixed and paraffin-embedded tissues of SCC of the UDT: 92 cases (17 OCs and 75 ECs) diagnosed at Kagoshima University Hospital, Kagoshima, Japan during 1987-2005; 90 cases (48 OCs and 42 ECs) diagnosed at King Edward Medical University, Lahore, Pakistan during the period 1996-2002; and 55 cases (6 OCs and 49 ECs) diagnosed at Hospital Universitario del Valle in Cali, Colombia during 1996-2001. For 11 HPV-positive EC cases from Japan, additional formalin-fixed and paraffin-embedded tissues of the esophagus, with or without cancer cells, were analyzed. The histological classifications for OCs and ECs were made using the guidelines determined by Japan Society for Head and Neck Cancer^[13] and Japanese Society for Esophageal Diseases^[14], respectively. These Japanese classifications follow their corresponding WHO classifications.

DNA extraction

Five-micrometer-thick sections of each tissue, containing a minimum of 60% (typically 70%-90%) tumors cells, were prepared. In each tissue sample, 0.8 mL lemosol and 0.2 mL ethanol were added. Subsequently, the samples were washed with 1 mL ethanol. After centrifugation, the pellet was resuspended in digestion buffer (50 mmol/L Tris-HCl, pH 8.0, 1 mmol/L EDTA, pH 8.0, 0.5% Tween 20) containing 200 µg Proteinase K (Invitrogen, Carlsbad CA, United States) and incubated at 56 °C for 24 h.

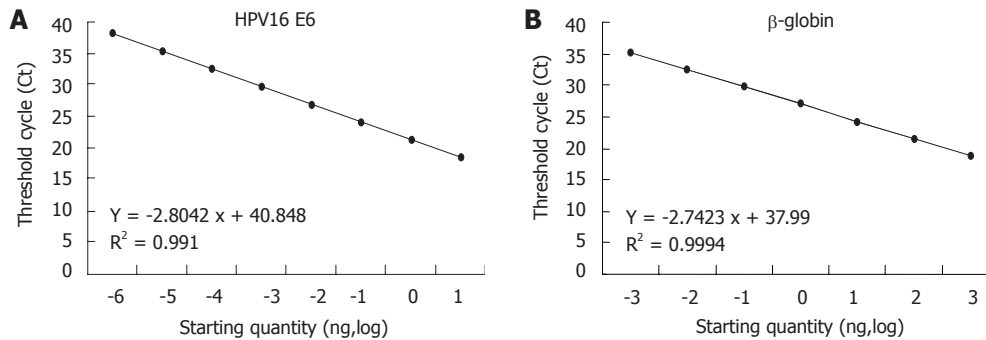


Figure 1 Real-time polymerase chain reaction standard curves. A: Human papillomavirus (HPV)-16 E6 DNA standard calibration curve was generated automatically by plotting Ct values against the logarithm of the copy numbers of eightfold serially diluted of HPV-16 cloned in pUC19 plasmid; B: A seven fold dilution series of a human DNA control (DynaL UK) was used to generate the standard curve for β -globin.

After incubation, the solution was heated at 100 °C for 10 min and centrifuged. Phenol-chloroform and DNA ethanol precipitation was made in all HPV16-positive samples in order to determine the DNA amount by using an ND-1000 spectrophotometer (Nano Drop Products, Wilmington, DE, United States). Since the quantity of tissue embedded in the paraffin blocks varied between samples, β -globin gene amplification was made for all the samples to check the presence of PCR amplification inhibitors and of amplifiable DNAs. The β -globin gene amplification with a set of PCO3/PCO4 primers^[15] was conducted under the following PCR conditions: initial denaturation at 95 °C for 4 min, 40 cycles with the cycling profile of 95 °C for 1 min, 52 °C for 1 min and 72 °C for 2 min, and final extension for 5 min at 72 °C.

HPV detection and genotyping

The prevalence of HPV DNA was analyzed with the broad-spectrum SPF1/2 HPV primers PCR method as described previously^[16]. The reaction was performed in a final volume of 25 μ L containing 3 μ L DNA template and 1.5 U AmpliTaq gold (PerkinElmer, Waltham, MA, United States). The mixture was incubated for 15 min at 95 °C, followed by 40 cycles of 1 min at 94 °C, 1 min at 45 °C, and 1 min at 72 °C, and a final extension of 5 min at 72 °C. The PCR products were run on a 3% agarose gel and the 65-bp product was visualized with ethidium bromide staining. The HPV types were determined using the INNO-LiPA HPV genotyping v2, which is based on the reverse hybridization principle. Part of the L1 gene region of the HPV genome was amplified using SPF10 forward and reverse primers tagged with a biotin at the 5' end, and denatured^[17]. Biotinylated amplicons were hybridized with specific oligonucleotide probes immobilized on the strip. In total, there were 25 genotypes (HPV6, 11, 16, 18, 31, 33, 35, 39, 40, 42-45, 51-54, 56, 58, 59, 66, 68, 70, 73 and 74). After hybridization and stringent washing, streptavidin-conjugated alkaline phosphatase was added and bound to any biotinylated hybrid previously formed. Incubation with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium chromogen gave a purple/brown precipitate and results could be interpreted visually.

HPV16 viral load

The quantitative real-time PCR analysis was performed with an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, United States). Each HPV16 DNA positive sample was amplified for 76 bp of the E2 gene using the following primers: forward 5'-AACGAAGTATCCTCTCCTGAAAT-TATTAG-3' (3361-3389 nt); reverse 5'-CCAAGGC-GACGGCTTTG-3' (3427-3443 nt), as well as 81 bp of the E6 gene, primers forward 5'-GAGAACTGCAAT-GTTTCAGGACC-3' (94-116 nt); reverse 5'-TGTATA-AGTTGTTTGCAGCTCTGTGC-3' (150-169 nt), in the presence of specific hybridization probes for E2-(FAM-CACCCCGCCGCGACCCATA-TAMRA) (3406-3424 nt) and E6-(FAM-CAGGAGCGACCCAGAAAGTTAC-CACAGTT-TAMRA) (119-147 nt). The reaction was performed in a 25 μ L mixture containing 1 \times TaqMan Master Mix (Applied Biosystems), 300 nmol primers, 100 nM dual-labeled E2 or E6 fluorogenic hybridization probe, and 1-2 μ L DNA template. Incubation for 10 min at 95 °C allowed activation of the AmpliTaq Gold DNA polymerase and denaturation of nucleic acids; 40 cycles of denaturation at 95 °C for 15 s and annealing-extension at 60 °C for 1 min were then carried out to amplify the E2 and E6 genes. Serial dilutions of full-length HPV16 genome cloned in pUC19 plasmid (kindly given by Dr. Massimo Tommasino, IARC, France) containing equivalent amounts of E2 and E6 genes from 86 to 862 million copies per reaction served as a standard control (Figure 1A). Each sample was assayed two or three times. DNAs extracted from SiHa cell SiHa cells was used as control for E2 (negative) and E6 (positive) amplification. This cell line derived from a cervical carcinoma is known to harbor one HPV16 genome or two per cell^[18]. Since this cell has only an integrated viral genome, its E2 gene is disrupted. To adjust for the differences in the amount of input genomic DNA between samples, quantitative real-time PCR for human β -globin gene was performed by 2 \times QuantiTect SYBR Green PCR kit (QIAGEN, Hilden, Germany) using the PCO3/PCO4 primers set^[15]. A sevenfold dilution series of a human DNA control (DynaL UK, Bromborough, Wirral, United Kingdom) was used to generate the

standard curve (Figure 1B). The amount of β -globin DNA present in each sample was divided by the weight of one genome equivalent (i.e. 6.6 pg/cell) and a factor of two (because there are two copies of β -globin DNA/genome equivalent or cell) to obtain the number of genome equivalents or cells in the sample. The viral load of each sample was expressed as the number of HPV16 copies per cell.

HPV16 physical status

The HPV16 physical status was determined on the assumption that the *E2* gene is disrupted in integrated viral genome, and therefore, the expected ratio of *E2* to *E6* copy numbers was zero. On the other hand, episomal viral genome had equivalent copy numbers of the *E2* and *E6* genes (an *E2/E6* ratio was nearly equal to unity) and mixed presence of integrated and episomal forms of HPV16 had an *E2/E6* ratio between 0 and 1^[19].

HPV16 E6 sequencing and variant analysis

HPV16 *E6* gene was divided into two fragments and amplified by two semi-nested PCRs, using outer primers 5'-TTGAACCGAAACCGGTTAGT-3' (forward, 46-66 nt) and 5'-GCATAAATCCCGAAAAGCAA-3' (reverse, 236-256 nt), and inner primers 5'-GCACCAAAAGAGAACTGCAA-3' (forward, 85-105 nt) for the first half. The outer primers of the second half were 5'-GGGATT-TATGCATAGTATATAGAGA-3' (forward, 246-270 nt) and 5'-CTTTGCTTTTGTCCAGATGTC-3' (reverse, 453-474 nt), and inner primers 5'-CAGGACACAGTGCTTTTGA-3' (reverse, 421-440 nt). The primers were designed using the web-based tool Primer3^[20]. Each reaction mix for *E6* amplification contained 5 μ L template DNA, 200 μ mol/L dNTP, 0.5 μ mol/L each primer, and 1 U Hot star Taq DNA polymerase (QIAGEN) in a total volume of 25 μ L reaction buffer (50 mmol/L KCl, 20 mmol/L Tris-HCl, pH 8.3). The first-round PCR condition was 95 °C for 15 min, followed by 40 cycles of 95 °C for 1 min, 55 °C for 1 min, and 74 °C for 1 min, and a final cycle of 74 °C for 10 min. The second-round PCR condition was essentially the same as the first round, except that 1 μ L of the first-round PCR products was used as template and that the number of PCR cycles was 35. The amplified products were confirmed through electrophoresis with 2% agarose gels at 100 V for 25 min. The positive amplicon was purified using the QIAGEN PCR purification kit and directly sequenced by fluorescent dye-labeled dideoxynucleotides and cycle sequencing methods using the Big Dye Terminator Cycle Sequencing Kit (PE Applied Biosystems, NJ, United States). The HPV16 *E6* sequences were aligned by CLUSTAL W multiple alignments package^[21], and compared with sequences of HPV16 variants that have been published elsewhere. HPV16 variants Genbank accession numbers were as follows: K02718, E-350T prototype; AF536179, E-350G variant; AF402678, Asian-American variant; AF534061, Asian variant and AF536180, African variant 1.

Immunohistochemistry for p16^{INK4a} and p53

The paraffin-embedded samples were cut in 2-3- μ m-thick slices, deposited on coated glass slides, and dewaxed using xylene. After rinsing with ethanol, the slides were incubated for 30 min in 0.3% H₂O₂/methanol and for 5 min in a microwave oven at 95 °C in 0.01 mol sodium phosphate/citrate buffer (pH 8.0). In order to block the nonspecific binding of the antibody, the slides were incubated for 30 min with 5% bovine serum albumin (BSA) in phosphate buffered saline (PBS) at room temperature. A 1:200 dilution in 5% BSA-PBS of monoclonal anti-p16^{INK4a} antibody was used (BD PharMingen, San Jose, CA, United States). The slides were incubated overnight at 4 °C, washed with PBS, incubated with biotinylated horse anti-mouse IgG for 30 min, and then washed with PBS and incubated with 1:50 dilution of the avidin-biotin-peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, United States) for 30 min at room temperature. The reaction was visualized by adding diaminobenzidine (Dako, Carpinteria, CA, United States) for 10 min. The sections were counterstained with hematoxylin and visualized. Immunostaining was considered negative when 0%-9% of the carcinoma cells were stained, and was considered positive when 10%-100% of the cells were stained, according to criteria reported previously^[22]. For p53, the procedure was the same as for p16^{INK4a}, but primary antibodies of p53 (1:50 dilution) was used (DO-7; Dako Japan, Kyoto, Japan). The interpretation of the positive signal was the same as that used for p16^{INK4a} immunostaining.

Statistical analysis

The χ^2 test, Fisher's exact test, Kruskal-Wallis test, and calculations of geometric mean of viral load and corresponding 95% confidence intervals were calculated with STATA software, version 9.2. (STATA Corp., College Station, TX, United States). All the *P* values presented were two sided.

RESULTS

The present study examined cases of SCC of the oral cavity (*n* = 71) and esophagus (*n* = 166) collected from Japan, Pakistan and Colombia. Sex distribution and mean ages by country are shown in Table 1. Although there was no sex difference in OCs among the countries, the proportion of male Japanese EC cases was higher than those of other countries. Pakistani OC and EC cases were younger than those in other countries.

The results of HPV detection using PCR with SPF1/2 consensus HPV primers are shown in Table 2. Although Colombian and Pakistani cases showed relatively higher HPV-positive rates in OCs and ECs, respectively, these differences were not statistically significant even after adjusting for sex and age distributions. In total, HPV was detected in 56% and 19% of SCCs of the oral cavity and the esophagus, respectively. The prevalence of HPV in

Table 1 Sex and age distribution of oral carcinoma and esophageal cancer cases

	Cancer site	
	Oral	Esophagus
Male/total (%)		
Japan	11/17 (65)	66/75 (88)
Pakistan	30/48 (63)	25/42 (59)
Colombia	4/6 (67)	27/49 (55)
¹ P value	0.972	< 0.001
Mean age in yr (95% confidence interval)		
Japan	68 (64, 73)	64 (62, 65)
Pakistan	49 (45, 53)	54 (50, 57)
Colombia	67 (61, 73)	64 (60, 67)
² P value	0.013	< 0.001

¹P values were obtained by χ^2 test. ²P values were obtained by one-way analysis of variance.

Table 2 Frequency of human papillomavirus DNA in tumors by cancer site and country *n* (%)

Cancer site	country	Total	HPV-positive	HPV16-positive	Multiple HPV
Oral cavity		71	40 (56)	39 (55)	0
Japan		17	8 (47)	7 (41)	0
Pakistan		48	27 (56)	27 (56)	0
Colombia		6	5 (83)	5 (83)	0
¹ P value			0.305	0.193	-
Esophageal		166	31 (19)	24 (14)	13 (8)
Japan		75	11 (15)	9 (12)	5 (7)
Pakistan		42	11 (26)	9 (21)	5 (12)
Colombia		49	9 (18)	6 (12)	3 (6)
¹ P value			0.308	0.331	0.521

¹Comparison among the three countries by χ^2 test. HPV: Human papillomavirus.

carcinomas was significantly higher in OCs than in ECs ($P < 0.001$), and similar trends were observed in all countries.

HPV16 was by far the most frequently detected HPV type. Except for one case, all HPV-positive SCC cases of the oral cavity harbored HPV16 only (39/40); the exception was an OC case in which HPV6 was detected. There were no multiple infections of different HPV types in a single carcinoma specimen among OCs (Table 2). Among 31 HPV-positive ECs, 24 were HPV16-positive. Table 3 lists 19 EC cases with HPV of a type other than HPV16; of these, 13 involved multiple infections of different HPV types. All multiple-infection cases except one involved combinations of HPV16 and other HPV genotypes (12/13). The exceptional case involved multiple infection with HPV51 and HPV68.

The physical status of HPV16 was determined by the ratio between copy numbers of the viral *E2* and *E6* genes, which were measured by real-time PCR. Among the 63 HPV16-positive cases examined, only four had HPV16 in the episomal form (two OCs and two ECs), as shown in Table 4. In eight carcinomas (three OCs and five ECs), the HPV16 genome was present in both the episomal and integrated forms. In the remaining cases, the viral

Table 3 List of esophageal carcinomas with human papillomavirus genome other than human papillomavirus 16

Country	Sex	Age (yr)	HPV16	Other HPV type
Japan	Male	49	-	6
	Male	66	-	51/68
	Female	64	+	18
	Male	67	+	51
	Female	75	+	51
Pakistan	Male	65	+	51
	Female	39	-	18
	Male	43	-	45
	Male	48	+	6
	Male	34	+	6
	Male	54	+	35
	Male	55	+	45
Colombia	Male	58	+	45
	Male	59	-	18
	Male	65	-	18
	Female	68	-	18
	Female	74	+	6
	Female	72	+	18
	Male	67	+	18

HPV: Human papillomavirus.

Table 4 Physical status and E6 variants of human papillomavirus 16 in Japan, Pakistan and Colombia

		<i>n</i> (%)				¹ P value
		All	Japan	Pakistan	Colombia	
Oral cavity	Physical status	39	7	27	5	0.147
	Episomal	2	0	2 (7)	0	
	Mixed	3	0	1 (4)	2 (40)	
HPV16 E6 variant	Integrated	34	7 (100)	24 (89)	3 (60)	0.048
	Prototype	6	3 (75)	3 (21)	0	
	E-350G	13	1 (25)	9 (64)	3 (60)	
	Asian	2	0	2 (14)	0	
	Asian-	2	0	0	2 (40)	
	American					
Esophagus	Physical status	24	9	9	6	0.017
	Episomal	2	0	0	2 (33)	
	Mixed	5	0	4 (44)	1 (17)	
	Integrated	17	9 (100)	5 (56)	3 (50)	
	Prototype	2	2 (40)	0	0	
HPV16 E6 variant	E-350G	8	3 (60)	5 (100)	0	< 0.001
	Asian	0	0	0	0	
	Asian-	6	0	0	6 (100)	
	American					

¹P values were obtained by Fisher's exact test. HPV: Human papillomavirus.

genome was considered to be present in the integrated form only. The frequency of HPV16 genome integration into the host genome was not related to sex, age or cancer site. However, the distribution of HPV16 physical status in ECs significantly differed among the three countries. All Japanese HPV16-positive cases showed an integrated form, but the frequency of integrated HPV16 was low in Pakistani and Colombian ECs.

HPV16 E6 variant analysis was also conducted (Table 4). The specimens in some cases had insufficient DNA, therefore, only 39 HPV16-positive cases were analyzed.

Table 5 Viral load of human papillomavirus 16 by country, physical status, and human papillomavirus 16 E6 variants

		<i>n</i>	Viral load/cell		
			GM	95% CI	¹ <i>P</i> value
Oral cavity Country	All	39	0.064	0.022, 0.185	0.033
	Japan	7	0.047	0.028, 0.079	
	Pakistan	27	0.037	0.010, 0.144	
	Colombia	5	1.883	0.058, 61.21	
Integrated form	Absent	2	0.001	-	0.048
	Present	37	0.081	0.028, 0.233	
E6 variant	<i>E-350G</i>	13	1.081	0.249, 4.705	0.038
	Others	10	0.127	0.063, 0.256	
Esophagus Country	All	24	0.121	0.053, 0.274	0.476
	Japan	9	0.072	0.023, 0.222	
	Pakistan	9	0.124	0.019, 0.799	
	Colombia	6	0.251	0.036, 1.737	
Integrated form	Absent	2	0.161	-	0.601
	Present	22	0.118	0.049, 0.285	
E6 variant	<i>E-350G</i>	8	0.444	0.101, 1.957	0.291
	Others	8	0.215	0.055, 0.847	

¹P values were obtained by Kruskal-Wallis test or Mann-Whitney U test. CI: Confidence interval; GM: Geometric means.

In Japan, only the *E-350T* prototype and the *E-350G* variant were detected. The predominant HPV16 variant was E-350G in Pakistani cases. In contrast, the Asian-American variant was more frequently found in Colombia, and these differences were statistically significant for both OCs and ECs ($P = 0.048$ and $P < 0.001$, respectively).

The geometric means of HPV16 were 0.064 and 0.121 per cell for OCs and ECs, respectively, and this difference was not statistically significant ($P = 0.552$). The HPV16 viral loads were also compared by country and the presence of the HPV16 integrated form and E-350G variant (Table 5). HPV16 in Colombian cases or cases with the E-350G variant tended to show higher viral loads in both OCs and ECs. The geometric means of the virus in OCs were 1.081, 0.147, 0.138 and 0.075 per cell for the E-350G variant, E-350T prototype, Asian-American variant, and Asian variant, respectively.

Comparison of *p16^{INK4a}* and p53 protein expression in HPV-positive and HPV-negative OCs and ECs suggested that the *p16^{INK4a}* expression was affected by the presence of the HPV genome in ECs (Table 6). However, the expression of these tumor suppressor genes was not related to HPV status in OCs.

It is difficult to deny the possibility that high-risk HPV was harbored in non-cancerous tissue adjacent to HPV-positive carcinoma, therefore, additional paraffin-embedded tissues of the 11 HPV-positive ECs from Japan were examined (Table 7). None of the normal esophageal epithelia adjacent to HPV-positive EC harbored a high-risk type of HPV genome.

DISCUSSION

The HPV genome was detected in 56% and 19% of SCCs of the oral cavity and esophagus, respectively, in

Table 6 Clinicopathological features of oral and esophageal carcinomas n (%)

Cancer site	Pathological features	HPV-negative	HPV-positive	¹ P value
Oral cavity	² Histological grading			0.899
	Well-differentiated	17 (81)	20 (77)	
	Moderate differentiation	3 (14)	5 (19)	
	Poor differentiation	1 (5)	1 (4)	
	³ p53			0.773
	Positive	13 (45)	16 (48)	
Esophagus	Negative	16 (55)	17 (52)	0.427
	⁴ p16 ^{INK4a}			
	Positive	11 (38)	10 (29)	
	Negative	18 (62)	25 (71)	
	² Histological grading			0.831
	Well-differentiated	42 (31)	10 (32)	
	Moderate differentiation	64 (48)	16 (52)	
	Poor differentiation	28 (21)	5 (16)	
	³ p53			0.368
	Positive	59 (44)	11 (35)	
	Negative	74 (56)	20 (65)	0.036
	⁴ p16 ^{INK4a}			
	Positive	42 (33)	16 (53)	
	Negative	86 (67)	14 (47)	

¹P values were obtained by χ^2 test. ²Information of histological grading was missing for 10 human papillomavirus (HPV)-negative and 14 HPV-positive oral carcinomas (OCs), and two HPV-negative esophageal carcinomas (ECs). ³Tissue specimens were not enough to examine p53 expression in two HPV-negative and seven HPV-positive OCs, and two HPV-negative ECs. ⁴Tissue specimens were not enough to examine p16^{INK4a} expression in two HPV-negative and five HPV-positive OCs, and seven HPV-negative and one HPV-positive ECs.

cases collected from Japan, Pakistan and Colombia. The HPV prevalence in both OCs and ECs did not significantly differ by country (Table 2). On the other hand, there was a significant geographical difference in the distribution of HPV16 E6 variants, which was also related to the viral load (Table 5). HPV16-positive OC cases with the E-350G variant showed a higher viral load than those with non-E-350G variants. Similar trends were observed in ECs although the difference was not statistically significant. One of the reasons for this difference is nucleotide alterations in primers and probes sequences. Among HPV16 intratypes, there is one polymorphism in the sequence of the E6 probe at nucleotide 145, and the Asian-American variant harbors this nucleotide substitution (C to T). However, this polymorphism is unlikely to cause a difference in viral load because the copy number of HPV16 in the Asian-American variant was similar to other intratypes except E-350G (data not shown). The HPV16 E-350G variant contains a polymorphism at residue 83, leucine to valine (L83V), which is associated with the risk of invasive cancers of the cervix in European studies^[23,24]. Yamada *et al*^[25] have identified five phylogenetic clusters of HPV16 with distinct geographic distributions by analyzing sequences of *E6*, *L1* and *LCR* regions isolated from cervical samples collected worldwide. HPV16 is the most prevalent HPV type detected in UDT cancer, therefore, different geographic distribution patterns of HPV16-E6 variants with differing copy num-

Table 7 Detection of human papillomavirus genome in adjacent normal epithelium of human papillomavirus 16-positive Japanese esophageal carcinomas

Case ID/sex/age	ID # of block	Histology	HPV	HPV type
EC4/M/48	39	SCC	+	16
	¹ 32	SCC	-	
	¹ 40	SCC	+	16
	14	Normal	-	
	37	Normal	-	
EC5/M/67	20	SCC	+	16, 51
	¹ 8	SCC	+	16
	¹ 14	SCC	+	6
	¹ 15	SCC	-	
	41	Normal	+	6
EC7/F/75	35	SCC	+	16, 51
	¹ 5	SCC	-	
	¹ 13	SCC	-	
	24	Normal	+	6
EC10/M/66	12	SCC	+	16
	¹ 6	SCC	-	
	20	Normal	-	
	22	Normal	-	
EC11/F/47	26	SCC	+	16
	¹ 18	SCC	-	
	¹ 10	SCC	+	6
	41	Normal	-	
EC16/M/65	55	SCC	+	16, 51
	55	Normal	+	6
EC28/M/61	12	SCC	+	16
	20	Normal	-	
EC35/F/64	25	SCC	+	16, 18
	28	Normal	-	
	33	Normal	-	
EC61/M/64	12	SCC	+	16
	28	Normal	-	
EC63/M/66	9	SCC	+	51, 68
	3	Dysplasia	-	
	11	Normal	-	
EC67/M/49	16	SCC	+	6
	¹ 5	SCC	-	
	1	Normal	-	

¹Additional esophageal carcinoma specimens analyzed for each patient. ID: Identification; SCC: Squamous cell carcinoma; EC: Esophageal carcinoma; HPV: Human papillomavirus.

bers might affect the detection rate of HPV genome if different methods for HPV detection were applied.

HPV involvement in UDT carcinogenesis is unclear. In the present study, a significant positive association between the presence of the HPV genome and p16^{INK4a} expression was observed in ECs but not in OCs (Table 6). In cervical carcinomas, p16^{INK4a} protein expression is known to be upregulated by HPV^[26], where retinoblastoma protein is inhibited by HPV E7 protein, causing the release of E2F protein, which in turn leads to p16^{INK4a} upregulation. Although the results of the present study suggest an etiological involvement of HPV in EC development, this association should be confirmed.

HPV detected in normal specimens or low-grade squamous intraepithelial lesions has been found not to be integrated into the host genome^[27]. However, it has been reported in many studies^[19,28] that HPV16 is often integrated into the host genome in cervical carcinomas and

is frequently accompanied by episomal HPV. These findings suggest an etiological involvement of HPV when integrated into the host genome. In most HPV16-positive cases in the present study, the viral DNA was integrated into the host genome. This finding is compatible with studies conducted in areas of high EC incidence in China, in which > 90% of high-risk HPV detected in ECs was found to be integrated into the host genome^[29,30].

In Japanese ECs, high-risk type HPV genomes were not detected in surrounding normal epithelial tissues of HPV-positive ECs (Table 7). This result is similar to a recent study from Australia^[31], showing that none of the 55 samples of normal esophageal squamous epithelium were HPV-positive. Although we cannot deny the possibility that tumor cells are more susceptible to the infection with high-risk type of HPV, these observations indicate a possible association between HPV infection and carcinogenesis in some ECs.

The geometric mean HPV16 load in ECs was 0.121 per cell in the current study. It is unlikely, however, that the low viral load was a consequence of formalin fixation because the parallel amplification of a housekeeping gene (*β-globin*) gave an estimate of the amount of amplifiable genomic DNA in individual samples. Our findings are compatible with Chinese studies on ECs that have reported a viral load of < 1 to 157 copies per cell^[32].

The prevalence of HPV was evidently higher in OCs than in ECs ($P < 0.001$). HPV typing analysis established that HPV16 was most common among SCCs with concomitant HPV infection. In fact, HPV16 was the only high-risk type detected in OCs. In ECs, although HPV16 was the most frequently detected, other high-risk HPV types such as HPV18, 45 and 51 were also detected. In this study, multiple infection with different HPV types was observed in 37% of HPV-positive ECs, but multiple infection was not found in OCs. Double infection with different HPV types in ECs has also been reported in a study from China^[33]. These findings are in contrast to those reported in a Japanese study that showed multiple-type HPV infection in 17/30 (56.7%) specimens of the oral cavity mucosa^[34].

Infection with multiple HPV types is not rare in cervical samples. Studies have shown that 10% or more of clinical lesions contain at least two different HPV types^[35,36]. Interestingly, the prevalence of multiple infections has been reported to decrease with increasing severity of cervical neoplasia^[37,38]. A study has shown that the frequency of multiple-type HPV infection is related to many factors, such as age and sexual behavior, as well as to variables affecting immune response, e.g., immunosuppressive conditions and *HLA* genotypes^[38]. In the present study, ECs with multiple-type HPV infection did not exhibit different clinicopathological features from ECs with single HPV infection. Silins *et al.*^[39] have suggested that infection with HPV6 might interfere with HPV16 in terms of cervical carcinogenesis. In the present study, there were three ECs with co-infection of HPV16 and HPV6. These cases did not have any common clinical features.

In a previous study of HPV16 variants using cervical specimens^[25], the Asian-American variant was isolated mainly from Central and South America and Spain. African variants 1 and 2, and the Asian variant were present mainly in samples from Africa and Southeast Asia, respectively. In all regions other than Africa, the E-350T prototype and the E-350G variant were detected. In the present study, only the E-350T prototype and the E-350G variant were detected in Japan. On the other hand, in Pakistan, E-350G was the predominant HPV16 variant. In Colombia, the Asian-American variant was the most commonly found type, but this variant was not found at all in Japan and Pakistan. Our findings are similar to those of Yamada *et al.*^[25], who detected the E-350G, E-350T, and Asian-American variants in 52%, 25%, and 20%, respectively, of 228 HPV16-positive cervical cancer specimens from Central and South America. No particular HPV16 E6 variant predisposed those infected to OCs or ECs.

One important question regarding the presence of HPV in UDT carcinomas is the route of HPV infection. HPV is known to be sexually transmitted in the case of the anogenital organs^[40], as well as in some cases of HPV infection of the oral cavity^[41]. In addition, several other possible routes of infection have been proposed for HPV infection of the oral and pharyngeal cavities. These include intrapartum infection during passage through an infected birth canal, transplacental infection *in utero* prior to birth, and postnatal infection by contact^[42,34]. For instance, HPV can be transmitted from a mother to her newborn baby during vaginal delivery, and this can result in recurrent respiratory papillomatosis. In addition, HPV DNA has been detected in the foreskin of normal newborns^[43], in a high percentage of neonates vaginally delivered by HPV-infected mothers, and in the amniotic fluid^[44]. These findings favor the mechanisms for HPV transmission at birth.

In the present study, HPV16 was frequently integrated into the host genome in patients with OCs and ECs. However, the viral loads in these malignancies were much lower than those found in cancer of the cervix. It should be noted, however, that human cancer can be regarded as a stem cell disease originating from a small fraction of cancer cells that show self-renewal and pluripotency and are capable of initiating and sustaining tumor growth^[45]. HPV may be present in only a small fraction of cancer cells with a stem cell-like nature present even in advanced tumors.

In conclusion, there was no significant difference of HPV prevalence in SCC of the UDT among populations at different risk of HPV exposure. The present study cannot deny a possibility of HPV16 involvement in the development of EC.

COMMENTS

Background

Human papilloma virus (HPV) is suspected to play causal roles in a variety of

human malignancies, mainly in cervical cancer. Recently, scientific studies have suggested an etiologic role HPV in a subset of cancers in the upper digestive tract (UDT), such as cancers of the oral cavity, oropharynx, larynx and esophagus. These studies have now recognized that HPV infection in oral cavity is a strong risk factor for head and neck squamous cell carcinoma; mostly for oropharyngeal cancer, but also for esophageal carcinoma, but this remains unclear and controversial.

Research frontiers

The evaluation of causality for the infectious agents as human carcinogens is difficult given their ubiquitous nature, the substantial length of time between infection and the cancer event, the nature of cofactors, and the rarity of malignancy among those infected. Thus, a central problem for the epidemiologist is to define the natural history of infection and to identify those factors that are related to the development of cancer. Hence, informative biomarkers of the agent (such as viral load), of the host (such as abnormal antibody pattern), and of other oncogenic exposures (such as tobacco use) are required for understanding the virus-human interactions and for developing interventions.

Innovations and breakthroughs

In the present study, cases of oral cavity and esophageal cancer were examined for concomitant HPV infection, the type of HPV involved, and multiple infection with different types of HPV in three countries, Japan, Pakistan and Colombia, with different risks of HPV exposure and genetic backgrounds, using the same methods. To shed light on the etiological significance of HPV in the development of oral cancer (OC) and esophageal cancer (EC), the viral load and physical status of HPV16 (which is the most commonly found HPV type worldwide) and HPV16-E6 variants were examined. Comparison of p53 and p16INK4a expression in HPV-positive and HPV-negative OCs and ECs was also made.

Applications

The prophylactic vaccine against HPV16 could prevent HPV16-associated malignancies if the vaccine were demonstrated to be capable of preventing oral HPV16 infection. Thus, these findings have created new potential opportunities for the primary prevention of other HPV-related malignancies.

Terminology

HPV is a member of the papillomavirus family, which is capable of infecting humans. Viral load is a measure of the severity of viral infection that can be calculated by estimating the amount of virus in a cell or tissue. Viral physical status: the HPV genome can be found in two physical states: integrated into the host genome or not integrated as an episomal molecule. E6 is the HPV protein associated with cancer development. p53 protein prevents cell growth and stimulates apoptosis in the presence of DNA damage. p16 protein is involved in tumor suppression.

Peer review

Currently, the HPV vaccines potentially hold promise for the prevention of a greater majority of HPV-positive cervical cancers in woman. Thus, studies that attempt to clarify the association between HPV with cancers of the oral cavity and esophagus are important. They give us reason to be optimistic that HPV vaccines may be protective against UDT HPV infection, and consequently, effective in preventing HPV-associated UDT cancers in both men and women.

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