Human papillomavirus infection and squamous cell carcinoma in Hong Kong: a case-control study

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KEY MESSAGES

1. Human papillomavirus (HPV) DNA was found in 1.5% to 7.9% of patients with oesophageal cancer in Hong Kong.
2. Viral phenotype and molecular markers did not suggest an association between HPV infection and the development of oesophageal cancer.
3. Preventative measures specific for HPV infection such as vaccination might not have an impact on controlling oesophageal cancer.

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Introduction

Globally, oesophageal cancer is the eighth most common cancer with an annual incidence of 456 000.[1] In Hong Kong, its annual incidence is 6.4 per 100 000 men and 1.3 per 100 000 women.[2] In different regions, the incidence can vary by >20-fold. The prevalence of human papillomavirus (HPV) in oesophageal cancer also varies widely and seems to be geographically linked. High-risk HPVs are associated with cervical, oropharyngeal, and anogenital cancers. Nonetheless, the role of HPV in oesophageal cancer remains in dispute. This study aimed to elucidate the role of HPV in oesophageal cancer in Hong Kong.

Methods

This multi-centre cross-sectional case-control study was conducted from April 2012 to March 2015 and was approved by the ethics committee of the participating hospitals. Patients with clinical indications who were scheduled for endoscopic examination were recruited. Those with oesophageal squamous cell carcinoma (SCC) were defined as cases, and those with non-malignant lesions or no abnormalities detected were defined as controls. Subjects with known primary cancer outside of the upper digestive tract or any known HPV-related cancers were excluded.

The quality of DNA extracted from tissue samples was assessed by amplifying a 509-bp fragment of the beta-globin gene. HPV DNA was detected by nested polymerase chain reaction (PGMY09/11 and GP5+/6+_HK52), and typed by sequencing. To analyse the oncogenic role of high-risk HPV, the most important E6/E7 mRNA species (E6*I) was measured. To assess RNA quality, extracted preparations were tested using quantitative reverse-transcription polymerase chain reaction (RT-qPCR) targeting the splicing region of mRNA encoded by the housekeeping gene RPS18.

The integration status of HPV was examined to determine the oncogenic role of HPV. Briefly, viral integration that disrupts the E2 ORF of the HPV genome results in loss of control of expression of viral oncogenes E6 and E7. By comparing the number of copies of E2 and E7 genes in a given sample, the physical status of the viral genome can be estimated. A SYBR Green-based quantitative qPCR method[3] was applied to quantify E2 and E7 gene levels. A 10-fold (≥3 threshold cycle) difference in genome copies between E2 and E7 was regarded as an indication of HPV integration.

The total viral load was measured by a SYBR Green-based quantitative qPCR targeting the E7 gene. To adjust for variation in the amount of cells collected in each sample, the normalised viral load was obtained as $(E7_{copy} / \beta\text{-actin}_{copy}) \times 2$, where $E7_{copy}$ is crude total viral load and $\beta\text{-actin}_{copy}$ is β-actin level.

The genomic DNA sequences that spanned exons 4-9 of TP53 were analysed. The CINtec $p16^{INK4a}$ monoclonal antibody Clone E6H4 (Ventana) was used for $p16^{INK4a}$ staining by immunohistochemistry.

Continuous variables were analysed using t test or Mann-Whitney *U* test. Categorical variables were assessed using Chi square or Fisher's exact test. A two-tailed P value of <0.05 was considered statistically significant.

Results

Of 166 patients with histologically confirmed oesophageal cancer, 143 (86.1%) were SCC, 20 (12.0%) were adenocarcinoma, one was adenosquamous cell carcinoma, one was non–small cell carcinoma, and

one was neuroendocrine carcinoma. Patients with SCC were regarded as cases. The 168 controls with no oesophageal cancer included those with gastritis (n=116, 69.0%), oesophagitis (n=22, 13.1%), polyp (n=2), candidiasis (n=1), ectopic gastric tissue (n=1), glycogenic acanthosis (n=1), herpes simplex ulcer (n=1), or intestinal metaplasia (n=1), as well as those with no abnormalities detected in the oesophagus and stomach (n=23, 13.7%). Of the 143 cases, 112 (80.1%) were male. Cancer patients were older than controls (65.0±10.0 vs 61.0±14.1 years, P=0.004).

HPV

A total of 14 samples were found to harbour HPV DNA: HPV16 (n=13) and HPV52 (n=1). The HPV DNA positive rate was comparable between cases and controls (3.5% vs 5.4%, P=0.622). Cases and controls with oesophageal samples positive for HPV were also comparable in age (61.7 vs 62.9 years, P=0.738) and male-to-female ratio (1.3:1 vs 1:1, P=0.646).

All five oesophageal SCC specimens that were positive for HPV DNA had an adequate quality of RNA, and none showed a positive signal after RT-qPCR targeting HPV16 E6*I mRNA.

All specimens showed a detectable level of E2 and were within 10-fold (ie 3 threshold cycle) of the corresponding E7 level derived from the same specimen, and none showed evidence of E2 disruption. A low-level viral load was obtained for the five HPV16 DNA-positive SCC specimens (0.06±0.04 viral copy / cell equivalent).

The five HPV-positive cases included four male smokers and one female non-smoker. TP53 mutations were found in two cases. One was a synonymous transition (CCC→CCT) at exon 5 codon 153. The other was a non-synonymous transition (TAT→TGT) at exon 6 codon 205. All these five cases were negative for $p16^{INK4a}$ staining, HPV16 E6*I mRNA was not detected, and no evidence of E2 disruption / viral integration was observed.

TP53 and $p16^{INK4a}$

A total of 48 (33.6%) of the 143 patients with oesophageal SCC had TP53 mutation. All had a single point mutation, and 38 (79.2%) also had a non-synonymous mutation. The most common

TABLE 1. TP53 mutation in subjects

Frequency of detection	Exon/ codon	Base substitution	Amino acid change	Transition/ transversion	CpG island	Within DNA-binding domain	Within L2/ L3/LSH	Disruptive	Truncating
6	4/37	TCC to ACC	S37T	Transversion	Non-CpG	No	No	No	No
1	4/125	ACG to ACT	No aa change	Transversion	CpG	Yes	Yes	No	No
1	5/138	GCC to GTC	A138V	Transversion	Non-CpG	Yes	No	No	No
1	5/153	CCC to CCT	No aa change	Transition	CpG	Yes	No	No	No
1	5/156	CGC to CGT	No aa change	Transition	CpG	Yes	No	No	No
1	5/157	GTC to TTC	V157F	Transversion	CpG	Yes	No	No	No
1	5/159	GCC to CCC	A159P	Transversion	CpG	Yes	No	No	No
4	5/175	CGC to CAC	R175H	Transition	CpG	Yes	Yes	No	No
7	5/176	TGC to TTC	C176F	Transversion	Non-CpG	Yes	Yes	Yes	No
1	5/176	TGC to TAC	C176Y	Transition	Non-CpG	Yes	Yes	No	No
1	6/192	CAG to TAG	Q192 stop codon	Transition	Non-CpG	Yes	Yes	Yes	Yes
1	6/193	CAT to TAT	H193Y	Transversion	Non-CpG	Yes	Yes	Yes	No
3	6/196	CGA to TGA	R196 stop codon	Transversion	CpG	Yes	No	Yes	Yes
3	6/205	TAT to TGT	Y205C	Transition	Non-CpG	Yes	No	No	No
1	7/245	GGC to TGC	G245C	Transversion	CpG	Yes	Yes	No	No
5	7/248	CGG to TGG	R248W	Transversion	CpG	Yes	Yes	Yes	No
8	7/249	AGG to AGA	No aa change	Transition	Non-CpG	Yes	Yes	No	No
1	8/266	GGA to GTA	G266V	Transversion	Non-CpG	Yes	No	No	No
9	8/273	CGT to CCT	R273P	Transversion	CpG	Yes	Yes	No	No
1	8/281	GAC to TAC	D281Y	Transversion	Non-CpG	Yes	Yes	No	No
2	8/282	CGG to TGG	R282W	Transversion	CpG	Yes	Yes	No	No
1	8/283	CGC to CCC	R283P	Transversion	CpG	Yes	Yes	No	No

mutation was the G:C→T:A transversion type (n=19, 39.6%), which has been reported to be associated with smoking. Nonetheless, mutation was not associated with the self-reported smoking history. The prevalence of G:C→T:A transversion in smokers and non-smokers was comparable (14.4% vs 12.5%, P=0.747). The 22 TP53 mutations were scattered through exons 4-8 and were more common in exons 5, 7, and 8 (Table 1). Two cases of SCC and two cases of adenocarcinoma were positive for $p16^{INK4a}$.

Smoking, drinking, and sexual history

Smokers were more common in cases than controls (69.0% vs 28.0%, P<0.001, Table 2). Drinking ≥3 glasses of wine or beer per week regularly was more also common in cases than controls (18.2% vs 2.4%, P<0.001). HPV status was not associated with smoking or drinking habit. Furthermore, HPV infection was not associated with oesophageal SCC even after adjusting for smoking and drinking habit (odds ratio [OR]=0.756, 95% confidence interval [CI]=0.216-2.641).

Cases and controls (9.1% vs 5.4%) as well as HPV DNA positive and negative subjects (14.3% vs 6.7%) were comparable in terms of a history of sexually transmitted disease (Table 3). Patients with oesophageal SCC were less likely than controls to report having only one or no sex partner in their lifetime (53.8% vs 72.6%, P<0.001), but were more likely to report having >10 sex partners in their lifetime (13.3% vs. 2.4%, P<0.001) [Table 3]. About 11.2% of cases reported having oral sex, and 10.5% did not respond to this question. These rates were similar to those for controls. Sexual history was not associated with HPV infection status.

HPV association with other characteristics

Respectively 80.0% and 78.3% of HPV DNA positive and negative patients with oesophageal SCC were male. Among controls, age was not associated with

TABLE 2. TP53 mutation, smoking, drinking, and sexual history according to disease and human papillomavirus (HPV) DNA status

Disease/HPV status	No. (%) of subjects		
	TP53 (non-synonymous) mutation	Ever Smokers	Regular drinkers (≥3 glasses of wine/beer per week)
Oesophageal squamous cell carcinoma (SCC) cases (n=143)	39 (27.3)	100 (69.9)	26 (18.2)
Non-cancer controls (n=168)	-	47 (28.0)	4 (2.4)
P value	-	<0.001	<0.001
HPV DNA positive (n=14)	-	5 (35.7)	1 (7.1)
HPV DNA negative (n=297)	-	142 (47.8)	29 (9.8)
P value	-	0.375	0.745
HPV DNA positive oesophageal SCC (n=5)	2 (40.0)	4 (80.0)	1 (20.0)
HPV DNA negative oesophageal SCC (n=138)	37 (26.8)	96 (69.6)	25 (18.1)
P value	0.614	1.000	1.000

TABLE 3. Sexual history according to disease and human papillomavirus (HPV) DNA status

Disease / HPV status	No. (%) of subjects					
	History of any sexually transmitted diseases	History of genital warts	Single or no sex partner in lifetime	>10 sex partner in lifetime	Ever had oral sex (yes)	Ever had oral sex (no response)
Oesophageal squamous cell carcinoma (SCC) cases (n=143)	13 (9.1)	2 (1.4)	77 (53.8)	19 (13.3)	16 (11.2)	15 (10.5)
Controls (n=168)	9 (5.4)	2 (1.2)	122 (72.6)	4 (2.4)	17 (10.1)	18 (10.7)
P value	0.201	0.871	0.001	<0.001	0.760	0.949
HPV DNA-positive (n=14)	2 (14.3)	1 (7.1)	11 (78.6)	1 (7.1)	1 (7.1)	1 (7.1)
HPV DNA-negative (n=297)	20 (6.7)	3 (1.0)	188 (63.3)	22 (7.4)	32 (10.8)	32 (10.8)
P value	0.281	0.047	0.245	0.971	0.666	0.666
HPV DNA-positive oesophageal SCC (n=5)	0 (0.0)	0 (0.0)	3 (60.0)	1 (20.0)	0 (0.0)	1 (20.0)
HPV DNA-negative oesophageal SCC (n=138)	13 (9.4)	2 (1.4)	74 (53.6)	18 (13.0)	16 (11.6)	14 (10.1)
P value	1.000	1.000	1.000	0.515	1.000	0.430

HPV status. All HPV found in cancer patients was HPV16, and also accounted for eight of nine HPV detected in controls. None of the HPV16 detected from cancer specimens showed evidence of viral integration, E6*I mRNA expression, or $p16^{INK4a}$ expression.

Discussion

In Hong Kong, the prevalence of HPV DNA in patients with oesophageal SCC was low (1.7-7.9%), which concurs with findings reported two decades ago.[4] None of our HPV-positive cancer samples showed viral integration or E6/7 mRNA expression. A similar prevalence (2.8-9.9%) of HPV DNA in non-cancerous oesophageal tissues also suggested that HPV was a bystander rather than the culprit. Furthermore, none of the HPV DNA-positive cancers exhibited $p16^{INK4a}$ overexpression, which is regarded as a surrogate marker for transcriptionally active E7.

Mutation status of tumour suppressor gene TP53 could be another surrogate marker of HPV involvement. E6 abrogates the function of p53, and thus bypasses the need for TP53 mutation. In this study, one of the five HPV-positive cancer samples had TP53 mutation. Although the prevalence of mutation appeared to be lower in the HPV-negative cancer group, the small number of HPV-positive cases makes comparison difficult. Of note, a study reported no association between TP53 mutation and HPV status in Hong Kong Chinese,[4] whereas another study reported a lower TP53 mutation rate in HPV-positive oesophageal cancer in Sichuan Chinese.[5]

HPV has been found in oesophageal cancer tissues for >15 years, yet its role in the aetiology remains debatable. It is plausible that certain geographically restricted environmental risk factors enhance the oncogenicity of HPV in oesophageal cells. Both higher and lower HPV positive rates are reported from areas with a high incidence of oesophageal cancer. Environmental carcinogens accumulate more preferentially in certain parts of the world.

Conclusions

In Hong Kong, HPV DNA was found in a small proportion of patients with oesophageal SCC. Virological and molecular analyses did not support an aetiological association. Preventative strategies specifically for HPV, including vaccination, might not affect the control of oesophageal cancer.

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