Association of Human Papillomavirus Type 58 Variant With the Risk of Cervical Cancer

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Human papillomavirus (HPV) type 58 has been found to be prevalent among Chinese patients with cervical cancer. This study examined the oncogenic risk of HPV58 variants in Hong Kong, a southern part of China. Altogether, 1924 women were studied: 42.8% with a normal cervix, 16.2% with cervical intraepithelial neoplasia (CIN) I, 12.7% with CIN II, 20.8% with CIN III, and 7.6% with invasive cervical cancer (ICC). The overall prevalence of HPV58 was 11.4% (220) and increased statistically significantly with the severity of neoplasia (P_{trend}<.001, χ^2 test for trend). Among HPV58-positive women, the occurrence of E7 632C→T (T20I) and E7 760G→A (G63S) variants (T20I/G63S) showed a positive trend of association with the severity of neoplasia (P_{trend}<.001, χ^2 test for trend). HPV58 variants carrying these two substitutions showed an odds ratio (OR) for ICC of 26.79 (95% confidence interval = 10.14 to 74.72), and this OR was 6.9-fold higher than the ORs of variants without these substitutions. Patients with CIN III or ICC who were also infected with T20I/G63S variants had a statis-

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tically significant younger age at diagnosis than those infected with other variants (median age = 37 years versus 48 years; P = .038, two-sided Mann–Whitney U test). Thus, HPV58 variants carrying E7 T20I/G63S substitutions may be associated with an increased risk for cervical cancer. [J Natl Cancer Inst 2002;94:1249–53]

The E6 and E7 proteins of human papillomaviruses (HPVs) have important functions in the development of cervical cancer, but the implications of sequence variations in E6 and E7 open reading frames (ORFs) are still being disputed *(1)*. Most studies on sequence variation have been based on HPV16, the most prevalent high-risk type of HPV *(2–5)*. Because our previous studies suggest that HPV58, a rare HPV variant worldwide, is prevalent in Hong Kong *(6,7)*, we examined sequence variations in E6 and E7 genes of HPV58 and their risk association with the development of cervical neoplasia.

Subjects were recruited from a colposcopy clinic and were classified according to biopsy examination findings. All subjects gave their written informed consent. Women found to have a normal cervix by colposcopy, hence not receiving a biopsy examination, were regarded as normal, provided that their subsequent Papanicolaou (Pap) smear taken 4–6 months later did not reveal neoplasia. Those women with abnormal or missing follow-up smears were excluded. Histologic findings of inflammatory changes or HPV effects but without neoplasia were also classified as normal. The study was approved by the local Institutional Ethics Committee.

The study included 1924 Chinese women, most of whom were descendants of families from Canton province in the southern part of China. Their mean age was 40.2 years (range = 16–88 years; standard deviation = 10.7 years). Diagnoses among these 1924 women were as follows: 823 (42.8%) with a normal cervix, 311 (16.2%) with cervical intraepithelial neoplasia (CIN) I, 244 (12.7%) with CIN II, 400 (20.8%) with CIN III, and 146 (7.6%) with invasive cervical cancer (ICC)—including 137 with squamous cell carcinoma, seven with adenocarcinoma, and two with adenosquamous carcinoma. Overall, 220 (11.4%) samples were positive for HPV58 by a type-specific polymerase chain reaction (PCR) that used primers E7-P1/E7-P2 (5′- CTGTAA CAACGCCATGAGAG-3′ and 5′-TCAGGGTCATCCATTGCAGA-3′). The positive rate for HPV58 showed a statistically significant trend of increase with the degree of neoplasia (5.1% for normal cervix, 12.2% for CIN I, 17.2% for CIN II, 13.0% for CIN III, and 31.5% for ICC; P_{trend}<.001 by the χ^2 test for trend; Epi Info 2000; Centers for Disease Control and Prevention, Atlanta, GA).

Sequences of E7 and E6 regions were obtained by PCR-based cycle sequencing with E7-P1/E7-P2 primers and E6-P1/E6-P2 primers (5′-GACCGAA ACCGGTGCATATA-3′ and 5′- TCT CATGGCGTTGTTACAGG-3′), respectively. Sequencing reactions were

		Open reading frame																			Predicted amino acid substitution	
		E6						E7													E6	E7
HPV58 variant	No. of isolates	1 8 7	2 0 3	3 0 7	3 6 7	3 8 8	5 3 8	5 9 9	6 3 2	6 9 4	7 2 6	7 4 4	7 5 6	7 6 0	7 6 1	7 9 3	7 9 8	8 0 1	8 0 3	8 4 0	8 5 2		
Prototype-like	26	C	G	C	C	A	A	G	C	G	T	T	T	G	G	A	C	C	T	C	T		
E6/E7-HK-1	68	-	-	**T**	-	-	-	-	-	**A**	-	G	-	-	**A**	-	-	-	-	-	-		G41R / G63D
E6/E7-HK-2	58	-	-	**T**	-	-	-	-	**T**	-	-	G	-	**A**	-	-	-	-	-	-	-		T20I / G63S
E6/E7-HK-3	12	-	-	-	-	**C**	-	-	-	-	-	G	-	-	-	-	-	-	**C**	-	-	K93N	V77A
E6/E7-HK-4	12	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	**C**	-	-		V77A
E6/E7-HK-5	10	-	-	-	-	**C**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	K93N	
E6/E7-HK-6	6	-	-	-	-	-	-	-	**T**	-	-	G	-	**A**	-	-	-	-	-	-	-		T20I / G63S
E6/E7-HK-7	4	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
E6/E7-HK-8	4	-	**C**	T	**A**	**C**	-	-	-	-	-	G	-	**A**	-	**G**	T	**A**	-	-	-	E32Q / D86E / K93N	G63S / T74A / D76E
E6/E7-HK-9	2	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-		
E6/E7-HK-10	2	-	-	T	-	-	-	**A**	**T**	-	-	G	-	**A**	-	-	-	-	-	-	-		R9K / T20I / G63S
E6/E7-HK-11	2	-	-	T	-	-	-	-	-	**A**	-	G	C	-	**A**	-	-	-	-	-	-		G41R / G63D
E6/E7-HK-12	2	-	-	T	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-		
E6/E7-HK-13	2	-	-	T	-	-	-	-	-	**A**	-	G	-	**C**	**A**	-	-	-	-	-	-		G41R / G63H
E6/E7-HK-14	2	-	-	-	-	**C**	-	-	-	**A**	-	G	-	-	**A**	-	-	-	-	-	-	K93N	G41R / G63D
E6/E7-HK-15	2	-	-	T	-	-	C	-	-	**A**	-	G	-	-	**A**	-	-	-	-	-	-		G41R / G63D
E6/E7-HK-16	2	-	-	-	-	-	-	-	-	**A**	-	G	-	-	**A**	-	-	-	-	-	-		G41R / G63D
E6/E7-HK-17	2	-	-	T	**A**	-	-	-	-	-	C	G	-	-	-	**G**	T	**A**	-	-	-	D86E	T74A / D76E
E6/E7-HK-18	2	T	-	T	**A**	-	-	-	-	-	-	G	-	-	-	**G**	T	**A**	-	T	C	D86E	T74A / D76E

Fig. 1. Nucleotide sequence variations in the E6 and E7 open reading frames (ORFs) of human papillomavirus (HPV) 58 detected in 220 Hong Kong women. HPV58 prototype (GenBank accession number NC_001443) was used as the reference. Nucleotide positions where variations were detected are written vertically across the top. Positions at which no variation was found are marked with dashes. **Lightface type** = a silent nucleotide variation; **boldface type** = a missense variation. The one-letter amino acid code is used. The position of amino acid change is stated numerically. The letter preceding this number refers to the reference amino acid, and the letter following it refers to its substitution. Prototype-like isolates refer to those without nucleotide sequence deviation from prototype over the E6 and E7 ORFs. GenBank accession numbers for sequences reported in this paper are AF478150–AF478167 for E6 sequences and AF478132–AF478149 for E7 sequences.

performed on the ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA), according to the manufacturer's protocol (BigDye Terminator Cycle sequencing kit; Applied Biosystems). All sequences were confirmed by sequencing from both directions.

The HPV58 prototype (GenBank accession no. NC_001443), isolated from a Japanese cervical cancer patient, was used as the reference *(8)*. Six nucleotide sequence variations were found over the 450-base-pair (bp) E6 ORF, and 14 were found over the 297-bp E7 ORF. E6 variations were scattered, whereas E7 variations were mainly found within the carboxyl-terminal residues (amino acids 41–89). Sequence variability was higher for E7 than for E6. Only 10 (4.5%) isolates showed one or more variations at E6, whereas 176 (80.0%) isolates showed one or more variations at E7. The maximum number of variations for any variant was four (0.9%) for E6 and six (2.0%) for E7. The average chance of having a nucleotide sequence deviation from prototype was two substitutions per 1000 bp for E6 and eight substitutions per 1000 bp for E7. Thus, three of six E6 substitutions and nine of 14 E7 substitutions were nonsynonymous (Fig. 1). Mutations generating a frameshift or a premature stop codon were not observed.

Of the 220 HPV58 isolates, 26 (11.8%) showed complete E6 and E7 sequence homology with the prototype and were thus referred to as "prototype-like." The remaining 194 isolates were grouped into 18 different variants and named according to their frequency of occurrence as E6/E7-HK-1 to E6/E7-HK-18, respectively (Fig. 1).

The 388A→C (K93N) E6 nucleotide substitution showed a statistically significant negative trend of association with the severity of neoplasia (P_{trend}<.001 by the exact test for trend; StatXact 4.0.1; Cytel Software Corp., Cambridge, MA), whereas 632C→T (T20I) and 760G→A (G63S) E7 nucleotide substitutions showed a statistically significant positive trend of association with the severity of neoplasia (P_{trend}<.001 by the exact test for trend) (Table 1, A). E7 T20I and E7 G63S amino acid substitutions were apparently linked, with both found in 66 isolates. Only four isolates from subjects with a normal cervix had the G63S substitution in the absence of T20I. No isolate carried the E7 T20I substitution in the absence of E7 G63S. The HPV58 E6/E7-HK-2 variant that carried the E7 T20I and G63S (T20I/G63S) substitu-

Table 1. Distribution of human papillomavirus (HPV) 58 variant infection status according to the degree of cervical neoplasia*

	No. of women (%)							
	Normal	CIN I	CIN II	CIN III	ICC	Total	OR (95% CI)†	P_{trend}‡
A) HPV58-positive subgroup§								
Predicted amino acid substitution‖								
E6 ORF, E32Q	4 (9.5)	0	0	0	0	4 (1.8)		—
E6 ORF, D86E	4 (9.5)	2 (5.3)	2 (4.8)	0	0	8 (3.6)		—
E6 ORF, K93N	12 (28.6)	8 (21.1)	6 (14.3)	2 (3.8)	0	28 (12.7)		<.001
E7 ORF, R9K	0	0	0	2 (3.8)	0	2 (0.9)		—
E7 ORF, T20I	6 (14.3)	6 (15.8)	6 (14.3)	24 (46.2)	24 (52.2)	66 (30.0)		<.001
E7 ORF, G41R	20 (47.6)	8 (21.1)	18 (42.9)	16 (30.8)	16 (34.8)	78 (35.5)		.456
E7 ORF, G63D	20 (47.6)	8 (21.1)	18 (42.9)	16 (30.8)	14 (30.4)	76 (34.5)		.251
E7 ORF, G63H	0	0	0	0	2 (4.3)	2 (0.9)		—
E7 ORF, G63S	10 (23.8)	6 (15.8)	6 (14.3)	24 (46.2)	24 (52.2)	70 (31.8)		<.001
E7 ORF, T74A	4 (9.5)	2 (5.3)	2 (4.8)	0	0	8 (3.6)		—
E7 ORF, D76E	4 (9.5)	2 (5.3)	2 (4.8)	0	0	8 (3.6)		—
E7 ORF, V77A	2 (4.8)	6 (15.8)	6 (14.3)	6 (11.5)	4 (8.7)	24 (10.9)		.820
HPV58 variant								
E6/E7-prototype-like¶	4 (9.5)	8 (21.1)	8 (19.0)	4 (7.7)	2 (4.3)	26 (11.8)		.139
E6/E7-HK-1	18 (42.9)	6 (15.8)	18 (42.9)	14 (26.9)	12 (26.1)	68 (30.9)		.280
E6/E7-HK-2	6 (14.3)	6 (15.8)	6 (14.3)	22 (42.3)	18 (39.1)	58 (26.4)		<.001
E6/E7-HK-3	2 (4.8)	4 (10.5)	6 (14.3)	0	0	12 (5.5)		—
E6/E7-HK-4	0	2 (5.3)	0	6 (11.5)	4 (8.7)	12 (5.5)		—
E6/E7-HK-5	4 (9.5)	4 (10.5)	0	2 (3.8)	0	10 (4.5)		—
B) Whole study population#								
HPV58-negative	781 (94.9)	273 (87.8)	202 (82.8)	348 (87.0)	100 (68.5)	1704 (88.6)	Not done	
HPV58-positive, all variants	42 (5.1)	38 (12.2)	42 (17.2	52 (13.0)	46 (31.5)	220 (11.4)	8.55 (5.23 to 14.02)	
HPV58 E7 T20I/G63S variants	6 (0.7)	6 (1.9)	6 (2.5)	24 (6.0)	24 (16.4)	66 (3.4)	26.79 (10.14 to 74.72)	
HPV58 non-E7 T20I/G63S variants	36 (4.4)	32 (10.3)	36 (14.8)	28 (7.0)	22 (15.1)	154 (8.0)	3.88 (2.13 to 7.05)	
HPV58 E6 K93N variants	12 (1.5)	8 (2.6)	6 (2.5)	2 (0.5)	0	28 (1.5)	0.00 (0.00 to 2.42)	

*CIN I, II, or III = cervical intraepithelial neoplasia grade I, II, or III, respectively; ICC = invasive cervical cancer; OR = odds ratio; CI = confidence interval; ORF = open reading frame.

†OR (95% CI) for ICC relative to normal group by the two-sided χ^2 test or the two-sided Fisher's exact test, as appropriate.

‡By the two-sided exact test (StatXact 4.0.1) for trend in proportion with increasing severity of cervical neoplasia. A dash indicates that numbers are too low for statistical analysis.

§Total number of women per group: Normal = 42; CIN I = 38; CIN II = 42; CIN III = 52; ICC = 46; Total = 220.

‖Women with isolates having more than one missense nucleotide variation are counted more than once. Position of amino acid change is stated numerically. The one-letter amino acid code is used. The letter preceding this number refers to the reference amino acid derived from the prototype sequence (GenBank accession number NC_001443), and the letter following it refers to its substitution.

¶Isolates without nucleotide sequence deviation from prototype over the E6 and E7 ORFs.

#Total number of women per group: Normal = 823; CIN I = 311; CIN II = 244; CIN III = 400; ICC = 146; Total = 1924.

tions was found to have a statistically significant positive trend of association with the severity of neoplasia (P_{trend}<.001 by the exact test for trend; Table 1, A).

Isolates were further grouped according to the presence of E6 K93N and E7 T20I/G63S substitutions. E6 K93N-positive isolates included variants HPV58 E6/E7-HK-3, -5, -8, and -14. E7 T20I/G63S-positive isolates included variants HPV58 E6/E7-HK-2, -6, and -10. Among the study subjects (all had an abnormal Pap smear), the presence of HPV58 (prototype-like or any variant) carried an odds ratio for ICC of 8.55 (95% CI = 5.23 to 14.02). The corresponding odds ratio for E7 T20I/G63S-positive variants was 26.79 (95% CI = 10.14 to 74.72), whereas that for variants without these substitutions was 3.88 (95% CI = 2.13 to 7.05; Table 1, B). The association was 6.9-fold higher for E7 T20I/G63S-positive variants than for variants without these substitutions. The E6 K93N substitution appeared to be more prevalent among women with a normal cervix or low-grade lesions (Table 1, A). However, when the whole study population was considered, variants carrying this substitution did not show any statistically significant positive or negative association with ICC (Table 1, B).

Among patients with CIN III or ICC, infection with E7 T20I/G63S variants was associated statistically significantly with a younger age at diagnosis than was infection with other variants (median age [interquartile range]: 37 years [30.5–59.3 years] versus 48 years [36.8–63.0 years], P = .038 by two-sided Mann–Whitney U test; SPSS 10.1.0; SPSS Inc., Chicago, IL). This observation is in line with a higher oncogenicity of E7 T20I/G63S.

Worldwide, HPV58 has been found in only 2% of cervical cancers *(9)*. In contrast, one third of the patients with cancer in the current study were positive for HPV58. This unusually high prevalence has also been reported in Chinese populations living in Shanghai *(10)*, Jiangxi *(11)*, and Taiwan *(12)*. HPV58 is closely related to HPV33 and is grouped with HPV16, -31, -33, -35, -52, and -67 under the same branch of an HPV phylogenetic tree *(13)*. We found that sequence variability of HPV58 variants was fourfold higher for E7 than for E6, in contrast with HPV16, where E6 is reported to be more variable *(2–5)*. The E7 protein has three domains: Conserved region (CR)-1 (amino-terminal 20 amino acids) and CR-2 (amino acids 21–40) are similar to adenovirus E1A *(14,15)*, and CR-3 (carboxyl-terminal amino acids 41–98) contains two zinc-binding motifs (Cys-Xaa-Xaa-Cys) that are involved in dimerization and protein stability *(16)*. We found two substitutions that were associated with a higher oncogenic risk. The first substitution (E7 T20I) is located at amino acid 20, close to the Leu-Xaa-Cys-Xaa-Glu domain that mediates association with the retinoblastoma protein and its related proteins, p107 and p130. The second substitution (E7 G63S) results in a change from glycine to serine. The E7 protein is phosphorylated on serine residues by casein kinase II *(17)*, and a positive association between phosphorylation rate and oncogenic potential has been found *(18)*. Thus, we suspected that the E7 G63S substitution might have created an additional phosphorylation site that confers increased transforming activity. However, our hypothesis was not supported by an artificial neural network-based computer prediction (NetPhos 2.0 [http://www.cbs.dtu.dk/services/NetPhos/]) *(19)*. The NetPhos prediction gave a very low score for Ser-63 compared with Ser-31 and Ser-32, which are phosphorylated by casein kinase II (phosphorylation scores determined by NetPhos were 0.002 for Ser-63, 0.977 for Ser-31, and 0.992 for Ser-32). In the NetPhos prediction, a score closer to unity indicates a higher chance of phosphorylation.

In summary, our study provides epidemiologic evidence that HPV58 variants carrying E7 T20I/G63S substitutions are associated with an increased risk for cervical cancer. The exact mechanism for the increased oncogenicity needs further investigation. Cosegregating sequence variations in other parts of the virus genome may also play an important role. Finally, because this is a cross-sectional study, the magnitude of the risk should be interpreted cautiously.

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NOTES

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