

## Human papillomavirus type 16 E6, E7, and L1 variants in cervical cancer in Indonesia, Suriname, and The Netherlands

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### Abstract

**Objective.** Human papillomavirus type 16 (HPV 16) has several intratypic variants, and some are associated with enhanced oncogenic potential. For risk determination as well as for future vaccine development, knowledge about variants is important. Regarding the geographical distribution of HPV variants and the lack of data from Indonesia and Suriname, we studied the prevalence of HPV 16 variants in cervical cancer in these high incidence countries. Data were compared with The Netherlands, a low-risk country.

**Methods.** DNA samples from 74 formalin-fixed paraffin-embedded HPV 16-positive cervical carcinomas from Indonesia (Java,  $N = 22$ ), Suriname ( $N = 25$ ), and The Netherlands ( $N = 27$ ) were amplified using primers specific for the E6, E7, and part of the L1 regions. Products were sequenced and analyzed.

**Results.** A specific Javanese variant, with mutations 666A in E7 and 6826T in L1, was found in 73% of the Indonesian samples, 56% having an additional mutation in the E6 open reading frame (ORF; 276G), giving the predicted amino acid change N58S. This Javanese variant was also found in three Surinamese samples, which reflects what could be expected from migration of Javanese people to Surinam. Other non-European variants were identified in Indonesian, Surinamese, and Dutch samples in 14%, 28%, and 19%, respectively.

**Conclusion.** The majority of the HPV 16-positive cervical cancers in Indonesia are caused by a specific intratypic variant that was rarely found before in other countries.

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**Keywords:** Human papillomavirus type 16; Variant; Cervical cancer

### Introduction

Cervical cancer is the second most prevalent female neoplasm, the incidence of which varies from 10/100,000 in many western nations to 40/100,000 in developing countries [1]. Part of this difference is accounted for by the lack of screening programs and lack of good medical

care. Also differences in sexual behavior and parity are important factors.

In Indonesia, cervical cancer is the most common malignancy in females, with an estimated incidence of 25–40 per 100,000 women per year [2–4]. However, we expect this number to be an underestimation, because only 25–30% of all diseased people enter medical facilities [5]. Cervical cancer concerns a major health problem in Indonesia because most patients present in late stages of the disease in low resource settings where no screening program is available.

Suriname is another high-risk country, with an age standardized incidence rate of 26.7 per 100,000 women.

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Suriname has a very heterogeneous population, with many ethnic groups, some of which, Amerindians and Javanese, were described to be more likely to develop cervical cancer [6].

In contrast, the Netherlands is a low-risk country with an incidence of cervical cancer of 6.2 per 100,000 women per year. We used data from the Netherlands as representative of Western European countries [6].

Human papillomavirus (HPV) infection is the central cause of cervical cancer [7]. This DNA virus acts by the expression of two viral oncoproteins E6 and E7. The oncoproteins form complexes with p53 and Rb, respectively, leading to disruption of the cell cycle [8]. However, not all cervical infections with oncogenic HPV types will progress to cervical cancer. Factors that are described to be important in risk of progression to cancer are both of host and viral origin. One viral factor is the type and subtype of the papillomavirus involved. Almost 30 distinct types of HPV, oncogenic and non-oncogenic, have been described infecting the genital tract. HPV 16 and 18 are the most prevalent oncogenic types. HPV 16 is the most common, being associated with about 50% of cervical cancers [7].

Different types of HPV are defined as having more than 10% variation in specified regions of the genome [9]. Viruses differing by 2–10% are called subtypes and are infrequently observed, while those differing by <2% are more frequently seen and called intratypic variants. Because different types of HPV have been shown to have different oncogenic potential, it is reasonable to hypothesize that intratypic variants also have differential oncogenicity. Therefore, HPV intratypic variants, mainly of HPV 16, are extensively studied [10].

HPV 16 variants are described in phylogenetic branches, the distribution of which varies geographically. The prototype variant described by Seedorf et al. [11] is a German isolate and a member of the European branch. The other branches of variants that have been described are: Asian (As), mainly in southeast Asia; Asian–American (AA), mainly in Central and South America; the African-1 (Af1) and African-2 (Af2) variants that are mainly found in Africa; and the North-American variant (NA1) in America. The European variants are found in all other regions except for Africa. The E and the As branches are closely related, such that the As appears to be a subclass of the E lineage [12]. Because the HPV genome is very well conserved, one can reconstruct ancient spread of the virus and its co-evolution with humankind by studying the distribution on HPV variants [13,14].

Some authors have described a clear relationship between the risk of developing cancer and some non-European HPV 16 variants. However, contradicting results are found for HPV 16 variants within the European branch [8,10,15–21].

Apart from a worldwide study that includes some Indonesian samples in the East-Asian group [12], no data are available about the distribution of HPV 16 variants in

Indonesia or in Suriname. In this study, we describe the distribution of HPV 16 intratypic variants in these high-risk countries compared to the low-risk country of the Netherlands. We studied whether the prevalence of specific variants, associated with enhanced oncogenicity, may contribute to the high incidence of cervical cancer in these countries. Furthermore, information about the spread of HPV 16 variants may be important when developing an effective vaccine for the studied countries.

## Materials and methods

### *Specimen collection*

Samples from Indonesia were collected in the period from October, 2001 to March, 2002, from 74 subsequent patients newly diagnosed with cervical carcinoma visiting the National General Hospital “Dr. Cipto Manungkusumo” in Jakarta, Java.

Surinamese samples were obtained from 65 newly diagnosed cases of invasive cervical carcinoma from the Department of Pathology at the Academic Hospital of Paramaribo, Suriname, for the years 1989 through 1993, together with 51 patients visiting the same hospital in the same period who were referred for treatment to the Leiden University Hospital in Leiden, The Netherlands.

The Dutch group was obtained from 1989 to 1993 from 70 cervical carcinoma patients, all living in the Netherlands, treated in the Leiden University Hospital.

### *HPV typing*

The INNO-LiPA prototype research genotyping assay (DDL, Delft, the Netherlands), a highly sensitive reverse hybridization assay as previously described, was used for HPV genotyping [22].

We found 30 (41%) Indonesian, 42 (38%) Surinamese, and 34 (49%) Dutch samples to be HPV 16 positive. After exclusion of multiple infections, 24 Indonesian, 36 Surinamese, and 29 Dutch cervical carcinoma samples were further analyzed.

### *Variant identification*

DNA extracted from formalin-fixed paraffin-embedded material was used for amplification. Because formalin fixation causes fragmentation of DNA, we chose primers to amplify fragments of less than 250 bp. For the E6 open reading frame (ORF), we used three primer sets (E6 primer set 1: nt 46–65 5' -tgaaccgaaccggttagt-3', nt 237–256 5' -ttgcttttcgggatttatgc-3'; primer set 2: nt 204–224 5' -gcaacagttactgcgacgtg-3', nt 419–438 5' -tgtcaaaagccactgtgtcc-3'; primer set 3: nt 371–391 5' -cagcaatacaacaaccggtg-3', nt 568–590 5' -ggagatacactacattgcatga-3'). For the E7 ORF, we used two primer sets (primer set 1: nt 520–540

5'-ttgcagatcatcaagaacacg-3', nt 742–761 5'-tgtgactctagccttcggtt-3'; primer set 2: nt 691–710 5'-acaagcagaaccgga-cagag-3', nt 878–898 5'-caggtaccaatggggaag-3').

The following polymerase chain reaction program was used: 95°C for 5 min, followed by 40 cycles consisting of 30 s at 95°C, 45 s at 55°C, 60 s at 72°C, and an extension at 72°C for 7 min. To amplify the L1 ORF, we used the MY09/11 primer set as previously described [3]. Samples negative by the My-primer set were amplified by two primer sets covering the same area (L1 primer set 1: nt 6601–6619 5'-gcattgttgggtaacca-3'; nt 6781–6793 5'-tgtcatacgtctgcagtaagg-3'; primer set 2 nt 6782–6804 5'-ccttaactgcagcgtatgaca-3', nt 6970–6995 5'-ccttaattacttcccagaaagtg-3').

PCR products were tested on an ethidium bromide-stained 2% agarose gel. For positive products, a sequencing reaction was performed according to the manufacturer's protocol (BigDye Terminator Cycle sequencing kit; Applied Biosystems, Foster City, CA). Sequencing was performed separately with both forward and reverse primers. Only data with no discrepancies were used for analysis.

Samples were classified into phylogenetic branches from variation in the E6 and the My09/11 region of L1 region as described by Yamada et al. [12]. The sequence as published by Seedorf (HPV-16R) that belongs to the European lineage was used as a reference [11].

### Statistical analysis

We assessed the differences in distribution of variants between populations by using the Chi-square test.

## Results

DNA of E6, E7, and L1 was amplifiable and results were without discrepancies in 22/24 Indonesian, 25/36 Surinamese, and 27/29 Dutch HPV 16-positive cervical cancer samples. No frame shifts or premature stop codons were observed. All identified variants are listed in Fig. 1.

In the Dutch group, we found two new variants. These variants, A488C and G219A, were both found in the E6 ORF. The 219A mutation leads to the amino acid shift of arginine to glutamine at position 39.

The prototype variant (HPV 16-R) was detected in none of the Indonesian samples, but it was detected in 44% of the Surinamese and 41% of the Dutch samples. Non-European variants were found in Indonesian, Surinamese, and Dutch samples in 14%, 28%, and 19%, respectively. Asian variants were found in two Indonesian cases.

In the Indonesian group, the C6826T and G666A co-variation was found in 73% of Indonesian samples. In 56% of these, a third mutation was seen in the E6 ORF (A276G) that gives the N58S amino acid change. We will refer to the combination of the first two mutations, with or without the additional A276G, as the Javanese variant. In the Surinam-

ese group, this variant was found in three patients. It was not found in the Dutch group.

The distribution of variants in European, non-European, and the Javanese branch is depicted in Table 1.

The often-described European variant E-T350G in the E6 ORF was mostly found in the Dutch group (30%), and less in Surinamese (8%) and Indonesian (5%) samples.

In the E7 ORF, the only polymorphism that gives an amino acid change was from asparagine to serine at position 29 (N29S). In our group, this variation was found in 23% of the Indonesian and in 16% of the Surinamese patients. None of the Dutch samples contained this variant. It was linked to the prototype E6, the Javanese, the Asian, and also to some African variants (Table 2).

## Discussion

A remarkable finding in this study is the high percentage (73%) of a specific HPV 16 variant in Indonesian cervical cancer patients. This Javanese variant contains co-mutations in all three studied areas, namely C6826T in the L1 ORF, G666A in the E7 ORF, and in a large proportion of the cases (56%) also A276G in the E6 ORF. The variation in the E6 ORF leads to the amino acid change N58S. Because this is the first study of HPV variants in Indonesia and we only studied Java, this variant might also be prevalent in other areas of Indonesia.

All three variations in the Javanese HPV 16 variant were described before, but separately and only in small numbers. The Javanese variant, according to the phylogenetic branches yet described, will belong to the European branch. Although no definitions are set that determine when to introduce a new branch, the finding of variations in three different ORFs in a large number of the samples studied is an argument to define this variant as a separate branch.

Four different studies describe the A276G variant in E6 in small percentages (1–4%) in both cervical intraepithelial neoplasia (CIN) and cervical cancer. Two of these also describe the E7 ORF, and the E-A276G mutation was found both in combination with the G666A mutation as well as with a prototype E7 ORF [16,19,23,24]. The G666A variant in E7 was described by others in small numbers as well [20,23–25]. A study of the E7 ORF only among women in South China reports 3 out of 20 cervical carcinomas containing the G666A mutation [20]. Although a couple of papers describe HPV 16 variants in the L1 ORF, the C6826T variation is reported only in a worldwide study by Yamada et al. [12], including 35 samples from Southeast Asia. This variation was found in four isolates, two belonging to the European and two belonging to the African-2 branch. By using the same method, Wheeler et al. [3] described the My09/11 region and do not report the C6826T variation. Villa et al. [18] reported mutations in both the My09/11 area and in the E7 ORF in a Brazilian population but did not find any of the variations described by us.

Indonesia	Suriname	Netherlands	E6	E7	L1	Predicted substitution		
			1 1 1 1 1 1 1 1 1 1 1 2 2 2 2 3 3 3 4 4 5	6 6 7 7 7 8 8 8	6 6 6 6 6 6 6 6 6 6 6	E6	E7	L1
N=22	N=25	N=27	8 0 3 3 3 4 4 5 7 7 7 8 1 7 8 8 1 3 5 0 8 3	4 6 3 8 9 2 4 4	6 6 7 8 8 8 8 8 8 9 9	Class		
			3 9 1 2 5 3 5 3 3 6 8 8 9 6 6 9 0 5 0 3 8 2	7 6 2 9 5 2 3 6	8 9 2 0 1 2 5 6 6 7 9			
					9 5 1 3 5 6 4 2 5 0 4			
0	11**	11	A T A G A C G C C G T G G A T A T C T A A A	A G T T T A T T	T A G A T C C T C C G	HPV-16R		
1**	0	0	- c - - - - - - - C - - - - - - - - - - -	- - - - - - - - - - -	- - - - - - - - - - -	E-109c	E29Q	
0	1	0	- - G - - - - - - - - - - - - - - - - -	- - - - - - - - - - -	- - - - - - - c - - -	E-131G	R10G	
0	1	0	- - G - - - - - A - - - - - - - - - - -	- - - - - - - - - - -	- - - - - - - c - - -	E-131G	R10G, D25H	
0	0	1	- - - - - - - - A - - - - - - - - - - -	- - - - - - - - - - -	- - - - - - - - - - -	E-188A	E29K	
1	2	3	- - - - - - - - - - - - - - G - - - - -	- - - - - - - - - - -	- - - - - - - - - - -	E-350G	L83V	
0	0	1	- c - - - - - - - - - - - - - G - - - - -	- - - - - - - - - - -	- - - - - - - - - - -	E-350G	L83V	
0	0	1	- - - - - - - T - - - - - - - - G - - - -	- - - - - - - - - - -	- - - - - - - - - - -	E-350G	H24Y, L83V	
0	0	1	- - - - - - - - A - - - - - - - G - - - -	- - - - - - - - - - -	- - - - - - - - - - -	E-350G	E29K, L83V	
0	0	1	- - - - - - - - - - - A* - - - - - G - - -	- - - - - - - - - - -	- - - - - - - - - - -	E-350G	R39Q, L83V	
0	0	1	- - - - - - - - - - - - - - T G - - - -	- - - - - - - - - - -	- - - - - - - - - - -	E-350G	F69Y, L83V	
0	0	1	- - - - - - - - - - - - - - - - c* - - -	- - - - - - - - - - -	- - - - - - - - - - -	E-488c		
1	0	0	- - - - - - - - - - - - - - - - - - - G -	G - - - - - c - - -	- - - - - t - - - - -	E-647G		N29S
0	0	1	- - - - - - - - - - - - - - - - - - - g -	- - - - - g - - - - -	- - - - - - - - - - -	E-822g		
1	0	0	- - - - - - - - G - - - - - - - - - - -	G - - - - - c - - -	- - - - - - - - - - -	As-647G	D25E	N29S
1	0	0	- - - - - - - - G - - - - - - - - - - -	G - - - - - c c - -	- - - - - - - - - - -	As-647G	D25E	N29S
1	0	0	- c - - - - - - - - - - - - - - - - - - - a	- a - - - - - - - - -	G - - - - t - - - -	Java		S351A
2	0	0	- - - - - - - - - - - - - - - - - - - a	- a - - - - - - - - -	G - - - - t - - - -	Java		S351A
4	0	0	- - - - - - - - - - - - - - - - - - - a	- a - - - - - - - - -	- - - - - t - - - - -	Java		
0	1	0	- - - - C - - - - - - - - - - - - - - - - a	- a - - - - - - - - -	- - - - - t - - - - -	Java-135C	K11T	
1	0	0	- - - - - - - - A - - - G - - - - - - - - a	- a - - - - - G - - -	G - - - - t - - - -	Java-276G	D25H, N58S	S351A
6	2	0	- - - - - - - - - - - - G - - - - - - - - a	- a - - - - - - - - -	- - - - - t - - - - -	Java-276G	N58S	
1	0	0	- - - - - - - - - - - - G - - - - - - - - a	- a - - - - - - - - -	- - - - G t - - - - -	Java-276G	N58S	S393A
1	0	0	- - - - - - - - - - - - G - - - - - - - - G a	G a - - - - - - - - -	- - - - - t - - - - -	Java-276G	N58S,	N29S
0	1	0	c - - C - G T - - - - - a g G T - - - - G -	- c g - - - - - - -	- - a - - - t - - t a	Af1	R10T, Q14D, F69L, H78Y	N29S
0	1	0	c - - C - G T - - - - - a g - T - - - - - - c	- c g - - - - - - -	- - a - - - t - - t a	Af1	R10T, Q14D, H78Y	
0	0	1	- - - C - G T - - - - - a g - T - - - - - - c	- c g - - - - - - -	- - a - - - t - - t a	Af1	R10T, Q14D, H78Y	
1	2	0	- c - T - G T - - - - - a g - T - g - - - G -	- c g - - - - - - -	- C a - - - t - t t a	Af-2	R10I, Q14D, H78Y	N29S
0	0	1	- - - - - - T - - - - - a g - T G - - - - - c	- c g - - - - - - -	- C a - - - t - t t a	NA1	Q14H, H78Y, L83V	T353P
0	0	1	- - - - - - T - - - - - a g - T G - - g - - c	- c g - - - - - - -	- C a t - - t - t t a	AA	Q14H, H78Y, L83V	T353P
0	2	2	- - - - - T - - - - - a g - T G - - g - - c	- c g - - - - - - -	- C a t - - t - t t a	AA	Q14H, H78Y, L83V	T353P
0	1	0	- - - - - T T - - - - - a g - T G - - g - - c	- c g - - - - - - -	- C a t - - t - t t a	AA	Q14H, T17I, H78Y, L83V	T353P

Fig. 1. Sequence alterations relative to the E6, E7, and partial L1 open reading frame of the reference HPV 16 (HPV-16R) [11]. Phylogenetic lineages are noted as E for European, As for Asian, Java for Javanese, Af1 for African 1, Af2 for African-2, NA1 for North-American 1, and AA for Asian–American. For subclasses, the number and the letter represent the nucleotide change at that position. The capitals indicate variants with amino acid change. In the columns-predicted substitution, the letter preceding the amino acid position refers to the reference HPV 16 and the letter after refers to the substitution. \* denotes new mutation while \*\* denotes the L1 ORF of one sample could not be amplified.

Table 1  
Distribution of HPV 16 variants in Indonesia, Surinam, and the Netherlands

	Indonesia	Suriname	The Netherlands	
European	3	15	22	$P < 0.001$
Javanese	16	3	0	$P < 0.001$
Other Non-E	3	7	5	NS
Total	22	25	27	

Many studies have reported enhanced oncogenicity for specific HPV 16 variants [8,15–17]. Evidence exists mainly for non-European HPV 16 variants with some studies describing a 2- to 9-fold increased risk of cervical cancer [10]. Most convincing evidence was found in Mexico for the AA variant [21] and in Japan for the Asian variant [19]. In a study by Hildesheim et al. [26], it appeared that women in Costa Rica infected with non-European variants were also more likely to be diagnosed with cervical cancer compared to women infected with European HPV 16 variants. The Asian variant differs from the prototype by only one amino acid in E6 and is closely related to the European type. Nevertheless, it was convincingly described to be associated with enhanced oncogenicity [19]. Like other non-prototype variants, the Javanese variant we describe here might be associated with enhanced oncogenicity.

A variant often described in the literature is the HPV 16 European type with a base substitution at nucleotide 350 (T350G), resulting in the amino acid change L83V. Conflicting results have been reported concerning this variant. Some described it to be enriched in high-grade lesions. These studies were performed in English, Swedish, and Indian women [8,23,27,28]. In contrast, it was evenly distributed among high-grade CIN, low grade CIN, and cancer in a German, a Dutch, and a Chinese study [29–31]. In an Italian study, this variant was even associated with a lower risk for cervical cancer [24]. In our study, this variant was mostly found in the Dutch group and hardly in the high-risk countries.

The most reported amino acid change in the HPV 16 E7 ORF, the asparagine to serine mutation at position 29 (N29S), is likely to be significant because of its location in an immunoreactive region [23]. This mutation was mostly described in Asian countries. One survey among Korean women reports that this mutation was significantly more frequent in carcinomas (70%) compared to the control group (33%) or the CIN III group (50%) [25]. However, in Southern China and Japan, this variant was equally distributed among subjects [31,32]. In an Indian population, no associations were found between the E7 variant and tumor stage or age [28]. Altogether, data about the oncogenicity of this variant are contradictory. In our study, this mutation was found to be significantly more prevalent in the high-risk countries compared to the Netherlands ( $P \leq 0.05$ ).

The HPV 16 variants we found in Suriname can best be compared to the study of Yamada et al. [12] that provides data from several countries in Central and South America, not including Suriname. Comparing our Surinamese group

to Yamada et al.'s [12] data, these were rather different. The percentage of E-T350G variants in Suriname was only 8% compared to 52%, and the percentage of the Asian–American variant was 9% compared to 20% in published data. Both differences may be explained by the different immigration patterns in most Middle- and South-American countries compared to Suriname, where a large proportion of the population originates from India and Java [6]. Immigration from Java is also reflected in the fact that the Javanese variant as we describe in this study was also found in three samples from Suriname. The Javanese population in Suriname was described to have a higher incidence of cervical cancer compared to other ethnic groups in Suriname. Because the Javanese variant might be associated with enhanced oncogenicity, it is an interesting hypothesis that this difference in incidence might be explained by the prevalence of this variant in the Javanese population [6].

The data that exist about HPV 16 variants in the Netherlands were reported by Smits et al. [33], who found one E7 variant in five CIN lesions and three cervical carcinomas. This variant, A616T in a carcinoma patient, was not found in our group. Another study in the Netherlands only regarded the T350G nucleotide polymorphism [34]. In cervical carcinomas, they found the T350G nucleotide change in 44% of the cases compared to 30% in our study. Most of the polymorphisms we found have been described before. We identified two new variants, both in the Dutch group. No new variants were found in the scarcely studied areas in Suriname and Indonesia, where new variants were to be expected more readily than in the more frequently studied European population.

Because HPV types are different in oncogenic potential, it is reasonable to hypothesize that intratypic variants also have differential oncogenicity. High- and low-risk HPV types differ in their ability to degrade p53, so a difference in ability to bind and degrade p53 may also explain at least part of the difference in oncogenicity between intratypic variants [35].

In addition, some HPV variants may evade the natural immune response [15,10]. As we mentioned before, the oncogenicity of some variants seems to vary geographically and with the ethnicity of the studied population. This difference may be explained by the distribution of the highly polymorphic human leukocyte antigen (HLA) type, because some HLA alleles might more efficiently present specific epitopes from variants. Some studies have tried to identify whether specific variants are associated with different HLA

Table 2  
Rate of the amino acid change that was found in the HPV 16 E7 ORF compared between countries

	Indonesia	Suriname	The Netherlands	
N29S	5	4	0	$P < 0.05$
Prototype amino acid	17	21	27	
Total	22	25	27	

alleles [30,36–38], but the groups investigated were too small to provide conclusive results (reviewed by Hildesheim and Wang [10]).

Vaccines against HPV are under development. It is not clear to what extent cross-reactivity in immune response exists between HPV 16 intratypic variants, but knowledge about HPV variants may be important for vaccine design [39].

Additionally, when designing primers or probes in HPV-detecting methods or in research, one has to take the distribution of variants into account.

To clarify the significance of the proposed Javanese HPV 16 variant, additional data are needed concerning its possible enhanced oncogenicity and its geographical distribution.

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