

Human papillomavirus type 18 variants: Histopathology and E6/E7 polymorphisms in three countries

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In cervical cancer, human papillomavirus type 18 (HPV 18) and HPV 16 are predominantly related to adenocarcinomas (ADCs) and squamous cell carcinomas (SCCs), respectively. Here, we studied whether the geographically distributed HPV intratypic variants are also associated with histologically different tumors. A total of 44 HPV 18-positive and 91 HPV 16-positive cervical carcinomas from Indonesian, Surinamese and Dutch patients were histologically classified using hematoxylin and eosin, periodic acid Schiff plus and Alcian Blue staining. Samples were sequenced and intratypic variants were classified into the known phylogenetic branches. The Asian Amerindian HPV 18 variant was observed in 56% of ADCs compared to 15% of SCCs ($p < 0.006$). The African HPV 18 variant was exclusively found in SCCs. By sequencing the HPV 18 E6 and E7 open reading frames, we found predicted amino acid changes only in 8 samples. Two amino acid changes were consistent throughout the African branch. In HPV 16-positive tumors, we did not find a specific linkage between intratypic variants and histopathology. We conclude that HPV 18 intratypic variants are differentially associated with adenocarcinoma and squamous cell carcinoma of the cervix. The findings described here stress the biologic significance of intratypic HPV variants and might help explaining differences in the pathogenesis of cervical ADCs and SCCs.

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Infection with an oncogenic type of the human papillomavirus (HPV) is now established as the central cause of cervical cancer.¹ Almost 30 distinct types of HPV have been described infecting the genital tract. The oncogenic types HPV 16 and HPV 18 are the most prevalent in cervical cancer. The distribution of HPV types varies geographically, yet worldwide HPV 16 is present in 50% and HPV 18 in 14% of cervical cancers.² Indonesia is described as the only country where the number of HPV 16-positive cervical carcinomas is surpassed by the number of HPV 18-positive carcinomas, with a prevalence of 32% and 49%, respectively.²

Generally, HPV 16 predominates in squamous cell carcinoma (SCC), whereas HPV 18 is predominant in adenocarcinomas and adenosquamous carcinomas (ADCs).³ However, in previous studies, we found a difference in proportion of ADCs in HPV 18-positive tumors.^{4,5} In Indonesia, the proportion of ADCs in HPV 18-positive tumors was larger as compared to Suriname and the Netherlands. We hypothesize that this difference is to be explained by the geographical distribution of intratypic variants, that, similar to HPV types, might be preferentially associated with ADCs or SCCs.⁶ Two studies suggested an association between HPV intratypic variants and histopathology, but both included very small numbers of HPV 18-positive tumors.^{7,8}

HPV intratypic variants are defined as having less than 2% sequence variation compared to the prototype strain, whereas HPV types have more than 10% difference in nucleotide sequence and the infrequently observed subtypes differ by 2–10%.⁹ The intratypic variants are divided into phylogenetic branches. HPV 18 has 3 variant branches, a European, an African and an Asian Amerindian branch.¹⁰ HPV 16 is divided into 6 phylogenetic branches,⁹ plus the Javanese branch that we recently found to be prevalent in

a large number of Indonesian cervical carcinomas.¹¹ Some variants of HPV 16, the HPV type most investigated, have been associated with increased oncogenicity.⁹ Little is known about the oncogenicity of HPV 18 variants.¹²

In the present study, we assessed the distribution of intratypic HPV 18 and HPV 16 variants in ADCs and SCCs. Moreover, we did a sequence analysis on the E6 and E7 open reading frames (ORFs). We performed this study in patients from 3 different countries in order to investigate a wide range of ethnicities and variants. In the Indonesian group, a large majority of women are Javanese.⁵ The Surinamese population consists of several ethnic groups, the most important being the Creoles, Hindustani, Javanese, bush Negro and Amerindians.⁴ In the Netherlands, the majority of the population is Caucasian. We observed that HPV 18 intratypic variants are differentially distributed in ADCs and SCCs.

Material and methods

Study design

Indonesian samples were collected in the period from October 2001 to March 2002 from 74 subsequent patients newly diagnosed with cervical carcinoma visiting the outpatient clinic of the National General Hospital “Dr. Cipto Manungkusumo” in Jakarta, Indonesia, as described by Schellekens *et al.*⁵ These were extracted from a group of 104 first attendants with clinically a strong suspicion of cervical cancer. Thirty samples were excluded because tissue was not available or histopathologic examination did not reveal cervical carcinoma. Surinamese samples were 116 randomly selected newly diagnosed cervical carcinoma patients at the Academic Hospital of Paramaribo, Suriname, for the years 1989 through 1995. Among these patients were 51 who had been referred to the Leiden University Hospital in Leiden, the Netherlands, for surgical treatment. The Dutch group contained 105 randomly selected newly diagnosed cervical carcinoma patients, all inhabitants of the Netherlands, who visited the Leiden University Hospital from 1989 to 1995.

Histopathologic classification

An experienced pathologist histopathologically classified the tumors using hematoxylin and eosin (H&E), periodic acid Schiff plus (PAS+) and Alcian Blue staining procedures. We will refer to cervical cancers of glandular origin (adeno, adenosquamous and other rare glandular tumors) as ADCs.

Abbreviations: ADC, adenocarcinoma, adenosquamous carcinoma and other rare glandular tumors; H&E, hematoxylin and eosin; HPV, human papillomavirus; LCR, long control region; ORF, open reading frame; PAS+, periodic acid Schiff plus; SCC, squamous cell carcinoma.

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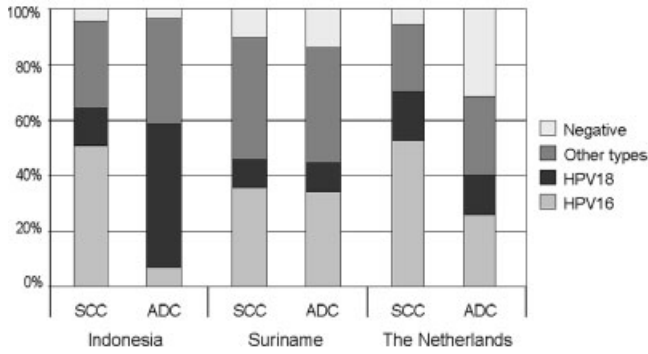


FIGURE 1 – Distribution of HPV 18 and HPV 16 in ADCs and SCCs in Indonesia, Suriname and the Netherlands. The category “other types” includes HPV types other than HPV 16 or HPV 18, and the multiple infections. In HPV 18-positive tumors, the distribution of ADCs and SCCs was significantly different between countries ($p = 0.009$, chi-square test). In HPV 16, no significant differences in distribution were observed ($p = 0.25$, chi-square test).

Human papillomavirus and variant typing

DNA extracted from formalin-fixed, paraffin-embedded tumor tissue was used for HPV type and variant analysis. To ensure lack of contamination, sections of a paraffin block without tissue, cut before each sample, served as negative control. All these controls were negative in the PCR analysis. INNO-LiPA prototype research genotyping assay (DDL, Delft, The Netherlands), a highly sensitive hybridization assay, was used for HPV typing as previously described.¹³ Part of the data on HPV typing in these samples was previously published.^{4,5}

HPV 18-positive samples were classified into phylogenetic branches on the basis of variation in part of the long control region (LCR) as described by Ong *et al.*¹⁰ As a reference, we used the HPV 18 sequence, published by Cole and Danos,¹⁴ that belongs to the Asian Amerindian lineage. For variant typing, we choose primers to amplify fragments of less than 350 bp, because formalin fixation causes fragmentation of the DNA. We designed 2 primer sets to amplify the HPV 18 LCR (LCR 1: nucleotides 7465–7483, 5'-TCGGTTGCCTTTGGCTTAT-3', and nucleotides 7753–7775, 3'-TGTC AACATTCTGTCTACCCTT-5'; LCR 2: nucleotides 7718–7738, 5'-GCTAATTGCATACTTGGCTTG-3', and nucleotides 144–163, 3'-CTACCTGATCTGTTCACGGGA-5'). The HPV 18 E6 and E7 ORFs were amplified by 3 different primer sets (set 1: nucleotides 89–118, 5'-AATACTATGGCGCGCTTGA-3', and nucleotides 428–447, 3'-CCAGAAACCGTTGAATC-CAG-5'; set 2: nucleotides 361–382, 5'-ATGGAGACACATTG-GAAAACT-3', and nucleotides 640–659, 3'-CAAAATGAA-ATTCCGGTG-5'; and set 3: nucleotides 601–623, 5'-TAAG-GCAACATTGCAAGACATTG-3', and nucleotides 912–930, 3'-CAATGGCTGATCCAGAAGG-5'). The following polymerase chain reaction program was used: 95°C for 5 min, followed by 40 cycles consisting of 30 sec at 95°C, 45 sec at 55°C, 60 sec at 72°C and an extension at 72°C for 7 min. Polymerase chain reaction products were tested on an ethidium bromide-stained 2% agarose gel and sequenced according to the manufacturer’s protocol (Big-Dye 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.

HPV 16-positive samples were classified into phylogenetic branches based on the nucleotide sequence of the E6 and the MY09/11 region of L1 as described by Yamada *et al.*⁶ Here we will focus only on the histopathology of HPV 16 variants, because previously we reported on sequence variation and distribution of HPV 16 intratypic variants in Indonesia, Suriname and the Netherlands.¹¹

TABLE I – DISTRIBUTION OF HPV 18 VARIANTS IN PHYLOGENETIC BRANCHES IN INDONESIA, SURINAME AND THE NETHERLANDS

	Asian Amerindian	European	African
Indonesia	9	8	0
Suriname	1	5	6
The Netherlands	4	10	0

*One variant that was phylogenetically located in between the European and the African branch and was observed in a Dutch patient was excluded. Data include only HPV 18 single infected tumors.

Results

Distribution HPV types

The number of HPV-positive samples was 71/74 in Indonesia, 103/116 in Suriname and 90/105 in the Netherlands. The distribution of HPV 16, 18 and other HPV types, including multiple infections, was found to be statistically different between countries ($p = 0.005$, chi-square test). The number of HPV 18-positive carcinomas including multiple infections was 28 (38%) in Indonesia, 20 (17%) in Suriname and 23 (22%) in the Netherlands. After exclusion of multiple infections, the histopathologic analysis was performed on the 21 Indonesian, 12 Surinamese and 17 Dutch HPV 18-positive single infected tumors. HPV 18 single positive tumors were ADCs in 71%, 25% and 29% in Indonesia, Suriname and The Netherlands, respectively, which was significantly different ($p = 0.009$, chi-square test). The distribution of the HPV types in ADCs and SCCs in each country is shown in Figure 1.

HPV 18 variants

Of the total of 50 HPV 18 single infected tumors, we were able to amplify the DNA and sequence the PCR product in 44 samples: 17/21 Indonesian, 12/12 Surinamese and 15/17 Dutch samples. In Indonesia, the Asian Amerindian variant was the most prevalent (9/17); in Suriname, this was the African variant (6/12); and in the Netherlands, the European variant was most commonly observed (10/14). The distribution of all HPV 18 variants in phylogenetic branches in each country is depicted in Table I. One variant, found in a Dutch patient, was excluded from Table I because it did not fit into 1 of 3 phylogenetic branches. This variant, described by Ong *et al.*¹⁰ as the T18-7, was phylogenetically located in between the European and the African branch.

The Asian Amerindian HPV 18 variant was significantly more often observed in ADCs compared to the other variants ($p = 0.005$, chi-square test). The African HPV 18 variant was exclusively related to SCCs, whereas the European variant had an intermediate distribution (Fig. 2). Also, when excluding the African variant from the analysis, the Asian Amerindian variant was significantly more often found in ADCs compared to the European variant ($p = 0.031$, chi-square test). When analyzing data from each country separately, the same trends were noticed, with a larger proportion of ADCs related to an Asian Amerindian variant as compared to the European variant, yet data were no longer significant (data not shown).

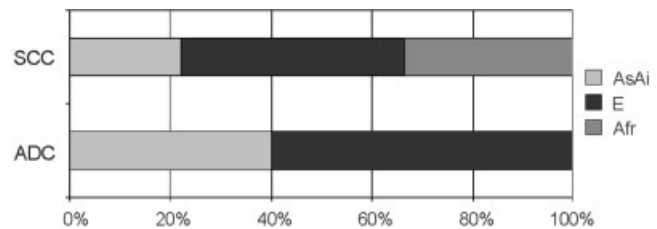


FIGURE 2 – Distribution of HPV 18 variants in ADCs and SCCs. Combined data from Indonesia, Suriname and the Netherlands. Asian Amerindian (AsAi) variant versus European (E) and African (Afr) variants ($p = 0.005$, chi-square test).

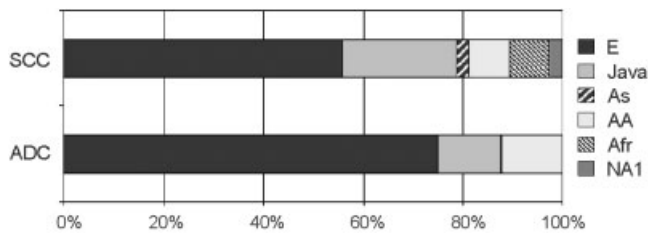


FIGURE 4 – Distribution of HPV 16 variants in ADCs and SCCs. Combined data from Indonesia, Suriname and The Netherlands. E, European; Java, Javanese¹¹; As, Asian; AA, Asian American; Afr, African; NA-1, North American 1.

response elements, and in HPV 16 it was demonstrated that at least 2 of these elements are located in the LCR.^{8,18,19}

An explanation for differences between HPV variants might also be derived from the E6 and E7 ORFs, because the E6 and E7 oncoproteins have essential functions in cell transformation and differentiation. Moreover, in HPV 16, many variations leading to amino acid changes were found that could possibly be responsible for different behavior of variants.

This is the first study to describe sequence variations in the HPV 18 E6 and E7 ORFs in clinical samples. We found covariations in the E6 and E7 ORFs of all nonprototype variants, but only 10 of 46 samples contained predicted amino acid changes compared to the reference sequence. Two of the substitutions were consistent throughout the 6 African branch variants. Yet no consistent differences in amino acid sequence were observed between the Asian Amerindian and the European branch. Therefore, we hypothesize that the observed differences in histopathology for the African branch might result from functional changes of the E6 or the E7 oncoprotein, which consequently leads to altered differentiation.

Yet they cannot explain the differences in histopathology between the European and the Asian Amerindian branches, which might be effects of variations in other parts of the viral genome, such as the LCR, as suggested by Burk *et al.*,⁸ or the E2 ORF, where large sequence variations were described by Hecht *et al.*²⁰

The relation between HPV 16 variants and histopathology was studied by Burk *et al.*⁸ They observed the non-European variants to be more frequent in ADCs compared to SCCs. The Asian American lineage of HPV 16 accounted for this difference. In our study, no significant differences in histopathology were observed between HPV 16 variants, but only 8 of 91 carcinomas contained the Asian American variant and these indeed were more frequently ADCs (25%) compared to tumors containing other HPV 16 variants. Some HPV 16 variants were never observed in ADCs, but numbers were too small to draw conclusions.

Just like HPV types, HPV intratypic variants have also been described to differ in their oncogenic potential, with most evidence existing for the HPV 16 Asian American variant.²¹ Some authors have suggested that HPV 18 variants also differ in oncogenic potential. Villa *et al.*¹² described that in Brazil the non-European HPV 18 variants persist more frequently and are more strongly associated with high-grade lesions compared to European variants. In addition, Hecht *et al.*²⁰ describe an HPV 18 subtype with decreased oncogenic potential. Further research is needed to assess the oncogenic potential of intratypic variants of HPV 18.

In conclusion, here we describe the association of HPV variants in relation to histopathology in the largest group of patients studied to date. We report that the Asian Amerindian and the African HPV 18 variant are strongly associated with ADCs and SCCs, respectively. Our group includes a relatively large number of HPV 18-positive tumors and contains a large proportion of non-European variants. The findings described here stress the biologic significance of intratypic HPV variants and might help explain differences in the pathogenesis of cervical ADCs and SCCs.

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