Chapter 5

Risks for persistence and progression by human papillomavirus type 16 variant lineages among a population-based sample of Danish women

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Abstract

Background

Little is known about factors determining HPV16 persistence and progression, but several studies have suggested that genetic variants may play a role.

Methods

HPV16-positive women with normal cytology in a large Danish cohort were reassessed for HPV16 status at 2 years and followed-up for cervical intraepithelial neoplasia 3 or worse (CIN3+) over 11 years through linkage with a national pathology database. Relative risks for clearance, persistence, and progression were compared with different HPV16 variant lineages based upon E6 gene sequencing.

Results

Sixty-two (23.7%) of 261 HPV16 infections were persistent at 2 years, and 32 (51.6%) persistent infections progressed to CIN3+. The majority of baseline infections belonged to the European lineage (97.3%), with EUR-350T and EUR-350G accounting for 61.3% and 36.0% of infections, respectively. At two years, the proportion of HPV16 infections that persisted was significantly higher for EUR-350T (28.2%) than EUR-350G (15.9%) variants (odds ratio = 2.06, 95% CI = 1.04–4.25). This increased risk for persistence was consistent both in the absence (OR = 2.16, 95% CI = 0.84–6.26) or presence (OR = 1.89, 95% CI = 0.76–5.15) of progression to CIN3+. Among persistent HPV16 infections, there was no significant difference in risk of progression to CIN3+ between EUR-350T and EUR-350G sublineages, which were both associated with a substantial absolute risk (>50%) of CIN3+.

Conclusions

Significant differences in risk for persistence exist between the HPV16 variants that predominate in Europe.

Impact

Understanding the genetic basis of HPV16 persistence and carcinogenicity may help unravel important interactions between HPV16 and the host immune system.

Introduction

Approximately 15 different human papillomavirus (HPV) types, referred to as high-risk (HR), are the aetiological agents of cervical cancer [39]. Although infection with a HR HPV is a relatively common event among sexually active women, the majority of infections are cleared by the immune system in 6–12 months [18]. Only a small percentage of infections persist and evolve into cervical intraepithelial neoplasia 3 (CIN3), which may progress to invasive cervical carcinoma (ICC) [19]. HPV16 is by far the most efficient HR HPV type in persisting [17,27] and causing progression into CIN3 or worse (CIN3+) [5,16,17].

Factors favoring HPV persistence *versus* clearance are still poorly understood, but several studies have suggested that intratype genetic variations may influence persistence and clinical outcome [2,7,9,22,23,26,28,32,33,38]. This phenomenon may be explained by natural variants that alter the immunogenic and/or carcinogenic properties of the virus. Many natural variants have been identified that differ from the original HPV16 prototype [29] in up to 2% of the coding region and/or 5% of the noncoding regions [1]. A number of studies have shown that the HPV16 E6 open reading frame is variable and is able to classify genomes into major phylogenetic lineages: European (EUR) that includes the arbitrary prototype sequence, Asian (As), Asian-American (AA), African 1 (AFR1), African 2 (AFR2), and North American (NA). The most common individual polymorphism in E6 is 350G that leads to a change from leucine to valine at codon 83 (L83V), which defines two distinct sublineages within the EUR lineage [30,36]. Some studies have suggested that the EUR-350G and EUR-350T sublineages vary in their distribution between cervical cancers and controls [11,37].

The present study takes advantage of a well-characterized population-based cohort study of 11,088 women [17] to determine whether specific variants of HPV16 E6 are preferentially associated with the natural history of HPV16 infection in Denmark.

Materials and Methods

Cohort

The population-based cohort has been previously described in detail [17]. In brief, between May 1991 to January 1993, 11,088 randomly selected women aged 20-29 years gave informed consent, underwent a personal interview, and gynaecologic examination including collection of endo-ectocervical cells for a Pap smear and HPV DNA detection (in phosphate buffered saline with 0.05% merthiolate). Between October 1993 and January 1995, the entire cohort was reinvited for a second examination (on average two years after the first), of which 8,656 women (78%) participated and underwent another personal interview. Endo-ectocervical cells were collected for a Pap smear and HPV DNA detection using the same procedures as at the first examination. All biological material was stored at -80°C.

In Denmark, every citizen has a unique 10-digit personal identification number (PIN). These identification numbers, which comprise information on sex and date of birth, are registered in the Central Personal Registry. The register is updated daily, and contains information on, e.g., vital status and migration including current address. This allows the correction of linkage with different registers and the possibility for a virtually complete follow-up.

All women were passively followed-up until February 2005 by linkage to the Danish Pathology Data Bank, using the PIN as key identifier for a median of 10.8 years, range 10.1 to 11.3 years. The Pathology Data Bank contains information on cervical screening history (organized and opportunistic), diagnoses of cytologic and histologic cervical abnormalities, and relevant treatment.

Study population

HR HPV positivity was evaluated by Hybrid Capture version 2 (HC2) (Digene) and all HR HPV-positive cervical specimens were typed using the line probe assay LiPa v2 (Innogenetics) in Germany, as described in detail previously [17]. A total of 355 women were HPV16-positive at the initial examination and

participated in the second active examination. Due to the poor quality of extracted DNA, 69 samples gave negative results in the PCR assay for the amplification of HPV16 E6 fragment and 25 samples from women with prevalent mild dysplasia or worse were excluded, leaving 261 HPV16-positive women with normal cytology included in the study, of which 62 were also HPV16-positive at the second visit. HPV16 variant status was determined from all HPV16-positive samples at the first and second examinations.

DNA extraction

DNA extraction was carried out using the MagnaPure device (Roche) using left over material from the HC2 assay. A PCR using primer set PC04/GH20, which amplified a 268-bp cellular beta-globin DNA fragment, was carried out for all the samples to ensure DNA quality [25].

Determination of HPV16 E6 variants

HPV16 E6 open reading frame was amplified by PCR with primers flanking outside of the entire coding region of HPV 16 E6 (nucleotides 52–575): 5'-CGAAACCGGTTAGTATAA-3' and 5'-GTATCTCCATGCATGATT-3' [37]. Forty amplification cycles were run in the GeneAmp PCR System 2400 with a 94°C denaturation step (1 minute), a 50°C annealing step (1 minute), and a 72°C extension step (1 minute), including an initial denaturation step of 15 minutes and a final extension step of 10 minutes resulting in a 524-bp product. The PCR mix contained 1X PCR buffer, 200 μmol/L of each dNTP, 0.2 μmol/L of each primer and 0.625 U of HotStarTaq DNA polymerase in a final volume of 25 μL (Qiagen). Five microliters of the PCR products were checked by ethidium bromide agarose gel electrophoresis. After enzymatic purification with 0.4 μL of Exonuclease I (10 U/μl) and 0.2 μl of Shrimp Alkaline Phosphatase (1 U/μl) at 37°C for 15 minutes and an inactivation step at 80°C for 15 minutes, the HPV16 E6 PCR products were sequenced by the fluorescent dye dideoxy termination method using an ABI Prism 377 DNA sequencer (PE Applied Biosystems). For

the sequencing reaction, the same primers were used as for the PCR reaction. The sequences were determined for both strands.

Statistical analysis

Study women were classified into three exclusive groups based upon the outcome of their HPV16 infection both at the second examination and during passive follow- up; (i) clearance, including all women who became HPV16 negative at the second examination, (ii) persistence no CIN3+, defined as women who remained HPV16 positive at the second examination, but who did not develop CIN3+, and (iii) persistence CIN3+, defined as women who remained HPV16 positive at the second examination and who developed CIN3+. Of note, 18 CIN3+ cases arose in women with cleared HPV16 infection (8 EUR-350T and 10 EUR-350G), but these CIN3+ cases were not studied separately due to the lack of data on the HPV type present in the CIN3+ biopsy. The relative risks of clearance, persistence, and progression were compared between different HPV16 E6 polymorphisms and lineages, using crude odds ratios (OR) and corresponding 95% confidence intervals (CI).

Results

Of the 261 HPV16-positive women with normal cytology at baseline, 199 cleared their infection and 62 persisted. Among the women with persistent infections, 32 (51.6%) developed CIN3+ during the ~11-year follow-up period (Table 1).

Table 1. Nucleotide sequence variations in E6 gene of 261 HPV16-positive Danish women, by outcome of HPV16 infection.

Status			HPV16 E6 nucleotide position[a]																										
Clearance N	Persistence N		109	131	132	135	143	145	173	176	178	185	187	212	215	216	219	256	267	285	286	289	315	335	350	403	532	(sub-) Lineage
	no CIN3+	CIN3 +	T	A	G	A	C	G	C	G	T	T	A	T	C	T	G	C	G	C	T	A	C	C	T	A	A	Reference
98	18	21	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	EUR-350T
4	2	1	-	-	-	-	-	-	-	-	-	-	g	-	-	-	-	-	-	-	-	-	-	-	-	-	-	EUR-350T
3	1	1	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	EUR-350T
2	0	0	-	-	-	-	-	-	-	-	-	-	-	c	-	-	-	-	-	-	-	-	-	-	-	-	-	EUR-350T
1	0	0	-	G	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	EUR-350T
0	1	0	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	EUR-350T
1	0	0	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	EUR-350T
1	0	0	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	EUR-350T
2	0	0	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	EUR-350T
1	0	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	EUR-350T
1[b]	0	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	EUR-350T
1	0	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	EUR-350T
62	7	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	EUR-350G
5	0	1	c	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	EUR-350G
5	0	1	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	EUR-350G
4	0	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	t	-	-	-	-	-	-	G	-	-	EUR-350G
1	0	0	c	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	EUR-350G
1	0	0	-	-	-	-	-	-	-	-	-	-	-	-	t	-	-	t	-	-	-	-	-	-	G	-	-	EUR-350G
1	0	0	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	EUR-350G
3	1	0	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	a	g	-	T	G	-	g	AA(non-EUR)
1	0	1	c	-	T	-	G	T	-	-	-	-	-	-	-	-	-	-	-	-	a	g	-	T	-	G	-	AFR2(non-EUR)
1	0	0	c	-	T	-	G	T	-	-	-	-	-	-	-	-	-	-	-	T	a	g	-	T	-	G	-	AFR2(non-EUR)
199	30	32																										

[a] Capital letters denote missense mutations, small letters denote silent mutations.
[b] Woman became infected with a new lineage 145T, 286a, 289g, 335T, 350G, 532g (AA) at second examination.
Abbreviations: EUR, EURopean; AA, Asian American; AFR, AFRican.

The large majority of HPV16 infections detected at baseline belonged to the EUR lineage (n = 254, 97.3%), with only four (1.5%) and three (1.1%) belonging to the AA and AFR2 lineages, respectively (Table 1). The EUR-350T sublineage accounted for 160 (61.3%) of infections at baseline, of which the HPV16 prototype E6 sequence was the most common genotype (n = 137) (Table 1, Figure 1). Other variants of the EUR-350T lineage were detected in seven (187g), five (131G), two (212c; 216G), or only one (131G/176A; 132A; 135C; 173T; 219A; 267A; 315G) woman. The EUR-350G sublineage accounted for 94 (36.0%) of infections at baseline (Figure 1), of which 350G in the absence of any other changes in E6 was the most common genotype (n = 74). Other variants of the EUR-350G lineage were detected in six (350G/109c; 350G/131G), five (350G/256t) or only one (350G/109c/185G; 350G/215t/256t; 350G/178G) woman (Table 1, Figure 1).

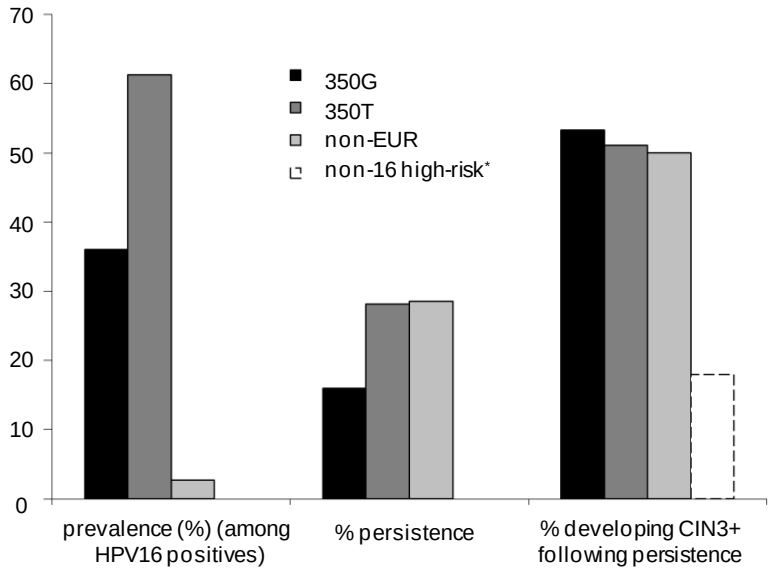

Figure 1. Prevalence of infection, percentage of women with persistent infection, and percentage of women with persistent infection who developed cervical intraepithelial neoplasia grade 3 or worse during follow-up, by HPV16 variant lineage. *Data from Kjaer and colleagues, JNCI 2010.

EUR-350G infections cleared in 84.0% of cases and persisted in 15.9% (Table 2). The respective proportions were 71.9% *versus* 28.2% for EUR-350T infections and 71.4% *versus* 28.6% for non-EUR (Table 2, Figure 1). The proportion of HPV16 persistent infections that developed into CIN3+ was similar by variant lineage (53.3%, 51.1%, and 50.0%, respectively). The statistical associations of HPV16 variant lineages with clearance, persistence and progression to CIN3+ are described in Table 2. EUR-350T infections were significantly more likely to persist than 350G infections (OR = 2.06, 95% CI = 1.04–4.25) (Table 2). When persistent infections were stratified by whether they progressed to CIN3+, the ORs for EUR-350T *versus* EUR-350G were similar in both strata, namely 2.16 (95% CI = 0.84–6.26) for persistence with no CIN3+ and 1.89 (95% CI, 0.76–5.15) for persistence with progression to CIN3. When restricted to the 62 women with persistent infections, there was no significant difference in risk of progression to CIN3+ between EUR-350T and EUR-350G sublineages (OR = 0.91, 95% CI = 0.24–3.46, data not shown).

Two out of the seven non-EUR HPV16 lineages persisted (14.3%), which is consistent with a greater risk for persistence in comparison with EUR-350G infections, but the difference was far from being statistically significant (OR = 1.98, 95% CI = 0.37–21.1) (Table 2).

When HPV16 sequences were dichotomized purely by the polymorphism at position 350, irrespective of lineage, infections carrying 350T (n = 163) were confirmed to be significantly more likely to persist than those carrying 350G (n = 98), with an OR of 2.01 (95% CI = 1.03–4.08). No other polymorphism was common enough to allow a statistical comparison using this approach.

Table 2. Outcome of HPV16 infection in 261 Danish women, by HPV16 variant lineage.

HPV16 status	N	EUR-350G		EUR-350T		Non-EUR		EUR-350T vs. EUR-350G		non-EUR vs. EUR-350G
		n	%	n	%	n	%	OR (95% CI)	OR (95% CI)	OR (95% CI)
Clearance	199	79	84.0	115	71.9	5	71.4	1.00	1.00	1.00
Persistence, no CIN3+	30	7	7.4	22	13.8	1	14.3	2.16 (0.84–6.26)	**2.06 (1.04–4.25)**	1.98 (0.37–21.1)
Persistence, CIN3+	32	8	8.5	23	14.4	1	14.3	1.89 (0.76–5.15)		
All		94		160		7				

Discussion

We report the first prospective study showing significant differences in natural history between the HPV16 variant lineages that predominate in Europe. This adds to the evidence from previous cohort studies showing that non-EUR HPV16 variants are more likely to persist and progress to CIN3+ than EUR HPV16 variants [28,32,35]. Our findings suggest that there is additional stratification of risk within the HPV16 EUR lineage so that, among Danish women at least, variants containing 350T (including the HPV16 prototype) have a higher risk of persistence and progression to CIN3+ than those containing 350G.

HPV16 infections circulating in the general Danish population were shown to be predominantly of the EUR variant lineage (97%), as seen in a previous population-based cohort study in France (95%) [11] and among series of HPV16-positive women referred for cervical abnormalities and/or diagnosed with cervical cancer in Northern Europe (90%–100%) [3,13-15,20,36,37], reviewed in[31]. This confirms findings that non-EUR (AA, As, AFR1, and AFR2) are far less frequent in Northern Europe than in Asia, Africa, or the Americas [36], reviewed in[31], and less common even than in Southern Europe, where 13%–36% of HPV16-positive women referred for colposcopy and/or diagnosed with cervical cancer have been reported as infected with non-EUR lineages [10,21,30,36,37].

The present population-based study is only the second in Europe to characterize the persistence and clinical outcome of HPV16 infection by variant status. In a previous study of 95 HPV16-positive women identified through routine screening in France [11], no statistically significant differences in risk for HPV16 persistence were identified by variant status. However, the definition of HPV16 persistence was less well defined than in the present study (i.e., persistent at 2 years) and 95% confidence intervals remain compatible with the present findings. EUR-350T accounted for 62% of all EUR lineages at baseline in Denmark, which compares with 52% in the French study [11] and with 45% among European women with normal cytology included in a recent literature review of cross-sectional studies [31].

Of note, the increased risk for CIN3+ associated with EUR-350T variants in the present study, although of borderline statistical significance, seemed to be primarily explained by the increased risk of persistence *per se*, with no evidence of additional excess risk of progression to CIN3 given persistent infection. This is similar to what has been reported for the differences in persistence and CIN3+ risk observed between non-EUR and EUR HPV16 variants in a previous cohort study [28].

Genetic variations in HPV16 may influence the prognosis of an infection in two ways, either by differing in their functional abilities or by evading the host's immune system. Given the fact that the increased risk seems stronger for persistence, rather than for progression to CIN3 given a persistent infection, the second option seems more likely. It is possible that HPV16 variants in concert with HLA [4] and other immune-genetic polymorphisms play a role in persistence. For instance, diverse HLA and killer immunoglobulin-like receptor combinations[24] or gene polymorphisms within non-coding regions of cytokine genes could confer susceptibility or be protective to cervical neoplasia development. Furthermore, the polymorphism at position 350 in E6 could be linked phylogenetically to one or more other parts of the HPV16 genome that are accounting for part, or even all, of the observed differences. Variations in these host characteristics could even account for any population-specific interactions with HPV16 variants, should they exist (see below).

Any true differences in risk for HPV16 persistence and CIN3+ by variant status would be expected to play out also for cervical cancer risk. However, although non-EUR HPV16 variant lineages, particularly AA, have been shown to be significantly overrepresented in cervical cancers compared with either populationbased [2,12] or clinic-based [30,38] controls, findings from case-control type comparisons of EUR-350T *versus* EUR-350G are less consistent. In two relatively small studies from Mexico and the Netherlands comparing cervical cancers and population-based controls, EUR-350T variants appeared overrepresented in cervical cancers in comparison with EUR-350G (ORs = 2.4, 95% CI = 0.3–115.0 and 1.7, 95% CI = 0.8–3.5, respectively), but differences

were not statistically significant. Other studies have compared cervical cancers only with clinic based controls referred for a broad range of nonmalignant cervical histopathologies, but at least one study has shown a significant excess of EUR-350T over EUR-350G in cervical cancers [6], whereas most have shown no significant differences in their relative distribution [14,15,20,30,38] or even suggest that the potential carcinogenicity of EUR-350T *versus* EUR-350G might be population-dependent [37].

If differences by population are confirmed to exist, they might be explained by residual genetic heterogeneity within HPV16 genomes classified solely upon position 350. Indeed, although the 350-position is highly polymorphic, it does not seems to robustly define phylogenetic lineages [8]. Alternatively, the oncogenicity of HPV16 variants also seem to differ by the ethnic origin of the host [34,35], suggesting the possibility of host variant interaction effects. Larger case-control studies with appropriate population-based controls are warranted in this regard.

The strengths of the present study include the population-based sample and the assessment of CIN3+ status over a long period of follow-up that had minimal scope to be biased by HPV variant status at study entry [17]. The principle limitation was the inability to make any robust reflection on the potentially increased carcinogenicity of non-EUR HPV16 lineages [32,35, 28], due to their rarity in the Danish population. However, the fact that two out of the seven women infected with HPV16 of non-EUR lineages at baseline developed CIN3+ (14%), is consistent with an elevated risk for these variants [2,12].

Among HPV16 infections that were persistent at 2 years, both EUR-350T and EUR-350G variants were associated with at least 50% risk of CIN3+ within 11 years, as reported previously for all HPV16-positive women in this cohort [17]. This compares with a figure of 18% for all non-HPV16 HR types in the same study (Figure 1). Indeed, a previous cohort study in France showed that both EUR-350T and EUR-350G variants were at increased risk of progression to CIN2/3 in comparison with all HR types other than HPV16 [11]. Thus, although the present study provides evidence that there are differences among the EUR

HPV16 lineage in persistence, it also shows that both EUR-350T and EUR-350G are associated with a substantial absolute risk of progression to CIN3+. Taking this evidence together with the fact that both lineages are found commonly in cervical cancers in Northern Europe [3,13-15,20,36,37], means that any clinical utility of variant analysis is not yet evident. Nevertheless, understanding the genetic basis of differences in the carcinogenicity of HPV16 variants, which may be driven by related sequence differences in other parts of the HPV16 genome, may help us unravel important biological and/or immunological interactions between virus and host that would lead to better tools to control HPV infection and its malignant consequences.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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