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HPV16 genetic variation and the development of cervical cancer worldwide

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Background: Factors that favour a small proportion of HPV16 infections to progress to cancer are still poorly understood, but several studies have implicated a role of HPV16 genetic variation.

Methods: To evaluate the association between HPV16 genetic variants and cervical cancer risk, we designed a multicentre case–control study based on HPV16-positive cervical samples (1121 cervical cancer cases and 400 controls) from the International Agency for Research on Cancer biobank. By sequencing the *E6* gene, HPV16 isolates were classified into variant lineages and the European (EUR)-lineage isolates were subclassified by the common polymorphism T350G.

Results: Incidence of variant lineages differed between cases and controls in Europe/Central Asia ($P = 0.006$, driven by an underrepresentation of African lineages in cases), and South/Central America ($P = 0.056$, driven by an overrepresentation of Asian American/North American lineages in cases). EUR-350G isolates were significantly underrepresented in cervical cancer in East Asia (odds ratio (OR) = 0.02 *vs* EUR-350T; 95% confidence interval (CI) = 0.00–0.37) and Europe/Central Asia (OR = 0.42; 95% CI = 0.27–0.64), whereas the opposite was true in South/Central America (OR = 4.69; 95% CI = 2.07–10.66).

Conclusion: We observed that the distribution of HPV16 variants worldwide, and their relative risks for cervical cancer appear to be population-dependent.

High-risk human papillomavirus (HPV) types are the aetiological agents of cervical cancer (zur Hausen, 2002), of which HPV16 is the most prevalent type worldwide, both in women without cervical abnormalities and in cervical cancer (Guan *et al*, 2012). Factors that favour a small proportion of HPV16 infections to progress to cancer are still poorly understood, but several studies have implicated a role of HPV16 genetic variation (Villa *et al*, 2000; Berumen *et al*, 2001; Sathish *et al*, 2005; Zuna *et al*, 2009; Schiffman *et al*, 2010; Gheit *et al*, 2011).

HPV16 variants have previously been classified into four major lineages based upon common phylogenetic patterns of single-nucleotide polymorphisms: (1) European Asian, including the sublineages European (EUR), and Asian (As), (2) African 1 (AFR1), (3) African 2 (AFR2) and (4) Asian American/North American (AA/NA), including the sublineages Asian American 1, Asian American 2 and North American (Yamada *et al*, 1995, 1997; Cornet *et al*, 2012). Other positions are frequently polymorphic within one or more lineages, but do not define phylogenetic sublineages (Chen *et al*, 2005). A common such polymorphism within the EUR lineage is T350G that is localised in the *E6* oncogene, and leads to an amino acid change from leucine to valine (L83V). Thus, the EUR lineage can be divided into isolates containing 350T (EUR-350T, which includes the prototype HPV16 sequence) or 350G (EUR-350G). This polymorphism has been suggested to influence the persistence and risk of progression to precancerous cervical lesions of EUR variant lineages (Zehbe *et al*, 2001; Grodzki *et al*, 2006; Gheit *et al*, 2011), and also occurs in non-EUR lineages.

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Using a multicentre case–control study design based upon HPV16-positive samples from the biobank at the International Agency for Research on Cancer (IARC), the aim of the current study was to evaluate the association between HPV16 genetic variants and the risk for cervical cancer in geographically and ethnically diverse populations of women worldwide.

MATERIALS AND METHODS

The IARC has coordinated cervical cancer case series, cervical cancer case–control studies and population-based HPV prevalence surveys in a large number of countries worldwide (Bosch *et al*, 1995; Muñoz *et al*, 2003; Clifford *et al*, 2005). All studies were approved by both local and IARC ethical committees. All cervical samples (exfoliated cells or tissue/biopsy specimens) from these studies have been genotyped for 37 HPV types using GP5+/6+-based PCR (Jacobs *et al*, 2000) in one centralised laboratory (Department of Molecular Pathology, Vrije University, Amsterdam, The Netherlands).

We designed a multicentre case–control study based upon the IARC biobank of HPV16-positive samples, with additional supplementation of HPV16-positive samples derived from cervical screening programmes in Italy (Sideri *et al*, 2009; Del Mistro *et al*, 2010) and Chile (Ferreccio *et al*, 2011). Cases were defined as women with HPV16-positive histologically confirmed cervical cancer, whereas controls included women with HPV16-positive normal cytology, atypical squamous cells of unknown significance or low-grade squamous intra-epithelial lesions. We included only countries ($n=15$) represented with both cases and controls in the IARC biobank.

For each selected HPV16-positive sample (exfoliated cells or tissue/biopsy specimens), DNA extraction, PCR amplification and sequencing of the entire HPV16 *E6* gene was performed at IARC, as previously described (Cornet *et al*, 2012). In brief, the primers used were flanking outside of the coding region of HPV16 *E6* (nucleotides 52–575): 5′-CGAAACCGGTTAGTATAA-3′ and 5′-GTATCTCCATGCATGATT-3′. Forty amplification cycles were run in the GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA, USA), resulting in a 524-bp E6 PCR product. After enzymatic purification with exonuclease I and shrimp alkaline phosphatase, the HPV16 PCR products were sequenced by the fluorescent dye dideoxy termination method using an ABI Prism 377 DNA sequencer (PE Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. For the sequencing reaction, the same primers were used as for the PCR. On the basis of the E6 sequence, HPV16 isolates were classified into four variant (sub)lineages (EUR, As, AFR, AA/NA) according to Cornet *et al* (2012). The EUR-lineage isolates were additionally stratified into EUR-350T or EUR-350G. No other non-lineage-specific polymorphism was frequent enough to be formally analysed. Countries were combined into regions, based upon geography and similarity of variant lineage incidence: North Africa, Sub-Saharan Africa, South/Central America, Eastern Asia, Western Asia and Europe/Central Asia. Because of the strong regional heterogeneity in the distribution of variant lineages (Table 1), regions were never combined in the following analyses.

Variant lineage distributions were compared between cases and controls using the Fisher's exact test. Associations between the EUR variants, EUR-350T and EUR-350G, and cervical cancer risk were estimated by odds ratios (OR) and 95% confidence intervals (CI), using the method of Mantel Haenszel, by country and by region (adjusted for country). The Breslow–Day test was used to test the homogeneity between crude ORs in South/Central America and other regions.

RESULTS

Valid E6 sequence data was obtained for a total of 1121 HPV16-positive cases and 400 HPV16-positive controls. Distribution of cases and controls by variant lineage and country/region is shown in Table 1. The EUR lineages accounted for the majority of isolates and were common in all regions except Sub-Saharan Africa and East Asia. The AA/NA lineages were detected in all regions, except Sub-Saharan Africa. The AFR lineages predominated in North and Sub-Saharan Africa, whereas the As lineage was specific for East Asia (Table 1).

In most regions, patterns of the HPV16 variant lineages did not differ significantly between cases and controls. However, variant lineages differed significantly between cases and controls in Europe/Central Asia (Fisher's exact test $P=0.006$), driven by an overrepresentation of AFR lineages in controls from Italy. There was also a difference of borderline statistical significance in South/Central America (Fisher's exact test, $P=0.056$), apparently driven by an overrepresentation of AA/NA isolates in cases.

The EUR isolates were further stratified into EUR-350T and EUR-350G, and their distribution compared between cases and controls for all regions, except sub-Saharan Africa (Table 2). The relative proportion of EUR-350T to EUR-350G among controls varied considerably by country/region (EUR-350T was more common than EUR-350G among cases in all regions, except South/Central America), as did their relationship with cervical cancer (Table 2). In South/Central America, the EUR-350G isolates were significantly overrepresented in cervical cancer compared with controls (OR = 4.69 *vs* EUR-350T; 95% CI = 2.07–10.66), an effect observed both in Argentina and Chile. In contrast, EUR-350G isolates were significantly underrepresented in cervical cancer in East Asia (OR = 0.02 *vs* EUR-350T; 95% CI = 0.00–0.37) and Europe/Central Asia (OR = 0.42 *vs* EUR-350T; 95% CI = 0.27–0.64), an effect that was consistent in all countries within those regions. The crude OR estimate for EUR-350G *vs* EUR-350T for South/Central America was significantly heterogeneous ($P<0.05$) from that of each of the other regions (data not shown).

In Sub-Saharan Africa, where the AFR lineages predominated, no differences were observed between cases and controls in the pattern of AFR sublineages [as defined by Cornet *et al* (2012), namely AFR1a (23 cases and 20 controls), AFR1b (0 and 1), AFR2a (18 and 18) and AFR2b (3 and 3); 5 other AFR isolates were not classifiable by AFR sub-lineage based upon *E6* alone; data not shown].

DISCUSSION

Using a multicentre case–control comparison based on HPV16-positive samples selected from the IARC biobank, we were able to identify significant associations between HPV16 variants and cervical cancer risk. Furthermore, we observed that the distribution of HPV variant lineages and EUR-350T/G worldwide, and the corresponding relative risks for cervical cancer, were population-dependent.

The distribution of major variant lineages around the world was confirmed to be highly geographically/ethnically specific (Yamada *et al*, 1997; Tornesello *et al*, 2004; Kang *et al*, 2005; Chimeddorj *et al*, 2008; Pande *et al*, 2008), limiting the possibility to compare their carcinogenic potential in a standardised way across all regions. However, our data did suggest an underrepresentation of AFR variants in cervical cancer cases in Europe/Central Asia, and possibly an overrepresentation of AA/NA variants in cervical cancer cases in South/Central America. Although these findings require further clarification, they do fit with the results of a study suggesting that HPV16 variants tend to persist and progress to

Table 1. Distribution of HPV16 variant (sub)lineages in cases and controls, by country and region

	Total	HPV16 lineages *N* cases/*N* controls				
Country/region	***N* cases/*N* controls**	**EUR**	**As**	**AFR**	**AA/NA**	***P*-value[a]**
Africa, North	*234/17*	*89/7*	*1/0*	*116/6*	*28/4*	*0.359*
Algeria	109/12	57/6	1/0	35/4	16/2	1.000
Morocco	125/5	32/1	—	81/2	12/2	0.126
Africa, Sub-Saharan	*43/50*	*1/1*	*0/0*	*42/49*	*0/0*	*1.000*
Guinea	40/25	1/0	—	39/25	—	1.000
Nigeria	3/25	0/1	—	3/24	—	1.000
America, South/Central	*102/44*	*86/43*	*0/0*	*2/0*	*14/1*	*0.056*
Chile	67/15	56/15	—	1/0	10/0	0.342
Argentina	35/29	30/28	—	1/0	4/1	0.366
Asia, East	*258/28*	*37/5*	*181/20*	*11/0*	*29/3*	*0.839*
Korea	47/4	7/2	35/2	—	5/0	0.181
Thailand	211/24	30/3	146/18	11/0	24/3	0.889
Asia, West	*151/75*	*136/67*	*2/1*	*1/0*	*12/7*	*0.940*
India	101/59	95/53	2/0	—	4/6	0.215
Nepal	24/12v	22/10	0/1	—	2/1	0.510
Pakistan	26/4v	19/4	—	1/0	6/0	0.612
Europe/Central Asia	*333/186*	*324/177*	*0/1*	*0/5*	*9/3*	*0.006*
Georgia	52/3	49/3	—	—	3/0	1.000
Italy	146/110	141/102	—	0/5	5/3	0.039
Poland	83/26	82/26	—	—	1/0	1.000
Mongolia	52/47	52/46	0/1	—	—	0.475
Total						
	1121/400	673/300	184/22	172/60	92/18	

Abbreviations: AA/NA = Asian American/North American; AFR = African; As = Asian; EUR = European.
[a]*P*-value of Fisher's exact test.

cervical intra-epithelial neoplasia grade 3 (CIN3) better in a host whose ethnicity shares an ancestral origin (Xi *et al*, 2007), even if this was not observed among greater racial admixtured populations of Latin America (Hildesheim *et al*, 2001; Sichero *et al*, 2007). The AA/NA variants have been previously associated with higher CIN3 and cervical cancer risk in studies from Costa Rica (Smith *et al*, 2011) and Mexico (Berumen *et al*, 2001), respectively, and with higher capacity for *in vitro* transformation of human keratinocytes (Sichero *et al*, 2012).

The EUR lineages accounted for a large proportion of HPV16 isolates in all regions, except Sub-Saharan Africa. Hence, analyses were well powered to study cervical cancer risks associated with the common EUR-350T/G polymorphism, revealing significant heterogeneity by world region; in Europe/Central Asia and East Asia, cervical cancer risk was significantly associated with the EUR-350T variants in comparison with EUR-350G. However, the opposite was true in South/Central America. Although puzzling, hints of this regional heterogeneity can be found in previous country-specific studies; EUR-350T variants were overrepresented in cervical cancers in comparison with EUR-350G in studies from both the Netherlands (Bontkes *et al*, 1998) and China (Chan *et al*, 2002), although no difference was seen in some other small series from Europe (Nindl *et al*, 1999; Hu *et al*, 2001a,b; Tornesello *et al*, 2004). Another study suggested that the carcinogenicity of EUR-350T *vs* EUR-350G might be population-dependent also within Europe (Zehbe *et al*, 2001). Furthermore, EUR-350T infections were more likely to persist and progress to CIN3 in comparison with EUR-350G in Denmark (Gheit *et al*, 2011). With respect to the opposite finding in South/Central America, a similarly strong association of EUR-350G with cervical cancer has been observed in a previous study from Argentina (Picconi *et al*, 2003), and a Brazilian study has recently reported a higher capacity for EUR-350G than EUR-350T variants to transform human keratinocytes *in vitro* (Sichero *et al*, 2012).

These differences by population might be explained by residual genetic heterogeneity within HPV16 genomes classified solely upon position 350, which, although highly polymorphic, does not seem to robustly define phylogenetic sublineages (Chen *et al*, 2005). Alternatively, host genetic factors, such as HLA haplotypes or TP53 polymorphisms, which differ by population, could have a role in the association between a particular HPV16 variant and cervical cancer development (Bontkes *et al*, 1998; van Duin *et al*, 2000; Zehbe *et al*, 2001). Whatever the underlying cause, such apparent geographical differences reveal an inherent complexity in studies of HPV16 variants and cervical cancer risk, and give a warning about the extent to which data can be pooled across countries/regions.

The availability of HPV16-positive controls, rather than HPV16-positive cases, is the limiting factor in the statistical power of this study and other studies of HPV16 variants and cervical cancer, given that they need to be derived from large population-based samples. This is particularly the cases in low-resource settings with no cervical screening programmes. Nevertheless, thanks to the reliance on 20 years of IARC studies on HPV and cancer from around the world, the number of HPV16-positive controls in the current study is the largest studied to date.

In conclusion, although our findings suggest that HPV16 variant analysis has no clinical application, understanding the genetic basis of differences in the carcinogenicity of HPV16 variants (which may be linked to genetic changes in non-E6 parts of the genome) may help us unravel important biological and/or immunological interactions between virus and host that could lead to better tools to control HPV infection and its malignant consequences.

Table 2. Distribution of EUR-350T and EUR-350G variants in cases and controls, by country and region

	Total	*N* cases/ *N* controls			
Country/ region	***N* cases/ *N* controls**	**EUR-350T**	**EUR-350G**	**OR[a]**	**(95% CI)**
Africa, North	*89/7*	*54/3*	*35/4*	*0.62*	*0.14–2.82*
Algeria	57/6	29/3	28/3	0.97	0.12–7.84
Morocco	32/1	25/0	7/1	—	—
America, South/Central	*86/43*	*19/26*	*67/17*	*4.69*	*2.07–10.66*
Chile	56/15	11/7	45/8	3.58	0.88–14.03
Argentina	30/28	8/19	22/9	5.81	1.64–21.12
Asia, East	*37/5*	*35/2*	*2/3*	*0.02*	*0.00–0.37*
Korea	7/2	7/1	0/1	—	—
Thailand	30/3	28/1	2/2	0.04	0.00–1.17
Asia, West	*133/67*	*36/25*	*97/42*	*1.61*	*0.86–3.02*
India	92/53	26/18	66/35	1.31	0.59–2.87
Nepal	22/10	5/5	17/5	3.40	0.52–21.94
Pakistan	19/4	5/2	14/2	2.80	0.15–46.47
Europe/ Central Asia	*324/177*	*156/65*	*168/112*	*0.42*	*0.27–0.64*
Georgia	49/3	12/0	37/3	0	0.00–4.37
Italy	141/102	60/29	81/73	0.54	0.30–0.96
Poland	82/26	39/5	43/21	0.26	0.07–0.82
Mongolia	52/46	45/31	7/15	0.32	0.10–0.97
Total[b]					
	669/299	300/121	369/178		

Abbreviations: CI = confidence interval; EUR = European; OR = odds ratio.
[a]Regional OR adjusted by country.
[b]Five EUR isolates (four cases and one control) with missing sequence information at nucleotide position 350 are excluded.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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APPENDIX

The members of the IARC HPV variant study group include previous IARC staff (N Muñoz, R Herrero, X Bosch) and local study coordinators (in alphabetical order by country): Algeria (D Hammouda); Argentina (D Loria, E Matos); Chile (C Ferreccio, F Aguayo); Georgia (T Alibegashvili, D Kordzaia); Guinea (N Keita, M Koulibaly); India (T Rajkumar, R Rajkumar); Italy (G Ronco, M Sideri); Korea (D-H Lee, H R Shin); Morocco (N Chaouki); Nepal (A Sherpa); Nigeria (JO Thomas, C Okolo, I Adewole); Mongolia (B Dondog); The Netherlands (CJLM Meijer, PJF Snijders); Pakistan (A Raza); Poland (W Zatonski); and Thailand (S Chichareon, S Sukvirach, S Tunsakul).