Genomic diversity of human papillomavirus-16, 18, 31, and 35 isolates in a Mexican population and relationship to European, African, and Native American variants

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Abstract

Cervical cancer, mainly caused by infection with human papillomaviruses (HPVs), is a major public health problem in Mexico. During a study of the prevalence of HPV types in northeastern Mexico, we identified, as expected from worldwide comparisons, HPV-16, 18, 31, and 35 as highly prevalent. It is well known that the genomes of HPV types differ geographically because of evolution linked to ethnic groups separated in prehistoric times. As HPV intra-type variation results in pathogenic differences, we analyzed genomic sequences of Mexican variants of these four HPV types. Among 112 HPV-16 samples, 14 contained European and 98 American Indian (AA) variants. This ratio is unexpected as people of European ethnicity predominate in this part of Mexico. Among 15 HPV-18 samples, 13 contained European and 2 African variants, the latter possibly due to migration of Africans to the Caribbean coast of Mexico. We constructed phylogenetic trees of HPV-31 and 35 variants, which have never been studied. Forty-six HPV-31 isolates from Mexico, Europe, Africa, and the United States (US) contained a total of 35 nucleotide exchanges in a 428-bp segment, with maximal distances between any two variants of 16 bp (3.7%), similar to those between HPV-16 variants. The HPV-31 variants formed two branches, one apparently the European, the other one an African branch. The European branch contained 13 of 29 Mexican isolates, the African branch 16 Mexican isolates. These may represent the HPV-31 variants of American Indians, as a 55% prevalence of African variants in Mexico seems incomprehensible. Twenty-seven HPV-35 samples from Mexico, Europe, Africa, and the US contained 11 mutations in a 893-bp segment with maximal distances between any two variants of only 5 mutations (0.6%), including a characteristic 16-bp insertion/deletion. These HPV-35 variants formed several phylogenetic clusters rather than two- or three-branched trees as HPV-16, 18, and 31. An HPV-35 variant typical for American Indians was not identifiable. Our research suggests type specific patterns of evolution and spread of HPV-16, 18, 31, and 35 both before and after the worldwide migrations of the last four centuries. The high prevalence of highly carcinogenic HPV-16 AA variants, and the extensive diversity of HPV-18, 31, and 35 variants with unknown pathogenic properties raise the possibility that HPV intra-type variation contributes to the high cervical cancer burden in Mexico.

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Introduction

Persistent infection with "high-risk" human papilloma-viruses (HPVs) is the primary cause of cervical cancer (IARC, 1995; Liaw et al., 1999; Munoz, 2000; Schiffman

HPV-16 VARIANTS

13%

14

98

87%

■ E □ AA

HPV-18 VARIANTS

13%

2

13

87%

□ E ■ Af1

Fig. 1. Prevalence of HPV-16 and HPV-18 variants in a cohort from Monterrey, Nuevo Leon, Mexico.

and Brinton, 1995; zur Hausen, 1996). Although early epidemiological studies identified only HPV-16 and HPV-18 as carcinogenic HPV types (see IARC, 1995, for a review), 17 HPV types closely or remotely related to HPV-16 are now considered as carcinogenic to the genital mucosa (Munoz et al., 2003). The prevalence of cervical cancer between different countries differs by about an order of magnitude. Generally speaking, developed countries have low and developing countries high incidences of cervical cancer. It is still poorly understood whether these disparities are mostly the result of access to better public health systems in developed nations, or whether divergent risk factors produce these different outcomes. Behavioral risk factors for developing cervical cancer that synergize with HPV infections include tobacco smoking, multiparity, use of anti-ovulants, and possibly nutrition (Castellsague and Munoz, 2003; Kjellberg et al., 2000; Moodley et al., 2003), and some of these, notably multiparity, differ between developed and developing countries.

Yet another possibility is that different cohorts are exposed to HPVs with differing pathogenic properties. Numerous epidemiological studies have confirmed that the same carcinogenic HPV types occur in all parts of the world (Bosch et al., 1995). HPV-16 is in nearly all countries of the world the most common HPV type, but the prevalence of other HPV types differs geographically. For example, HPV-45 is particularly common in West Africa, although HPV-39 predominates in Central and South America (Bosch et al., 1995). It is not clear whether such quantitative differences in the prevalence of HPV types contribute to the geographic disparities of the disease. However, it is well known that all HPV types occur in form of numerous genomic variants. These variants differ from one another by 1–5% of their nucleotide sequence (Ho et al., 1993; Ong et al., 1993; Stewart et al., 1996; Yamada et al., 1997), although by definition, the sequences between HPV types differ by at least 10% (Chan et al., 1995). Data suggest that variants of HPV-16 and possibly of other HPV types differ biologically and in their carcinogenic potential (Berumen et al., 2001; da

Reference	G	T	C	T	G	C	A	C	A	T	A	C	A	C	G	T	G	C	A	C	A	T	A	T	G	T	G	T	A	G	A	G	G	T	C
MX 851		G																																	
MX 617		G		C			A		G	G		C														G				G	T	C	A	T	G
MX 1125	C																																		
MX 990			C				A		G	G		C															A			G	T		A	T	
MX 80	C		C				A					C			-T	-G	-C	-A	-C	-A	-T					A				G	T		A		
MX 834			C				A		G	G		C															A			G	T		A	T	
MX 1144							A					C			-T	-G	-C	-A	-C	-A	-T					A				G	T		A		
MX 884	C		C				A		G	G		C										G					A			G	T		A	T	
MX 701			C				A					C			-T	-C	-A	-C	-A	-T						A				G	T		A		
MX 635												C			-T	-G	-C	-A	-C	-A	-T					A				G	T		A		A
MX 101															G		C					G					A						A		A
MX 933			C								T		A															G	A	G					
MX 974							A			G		C															A			G	T		A	T	
MX 812	G																					A													
MX 688			C				A		G	G		C															A			G	T		A	T	
MX 539																																			
MX 788										T		A													G	A	G								
MX 532			C				A		G			C															A			G	T		A	T	
MX 746			C				A		G			C															A			G	T		A	T	
MX 1241			C				A		G	G		C																		G	T		A	T	
MX 1108																																			
MX 580																																			
MX 1059			C								T		A												G	A	G								
MX 852																																			
MX 998									G			C															A			G	T		A	T	
MX 903						T																				A									
MX 889						T	A		G			C															A			G	T		A	T	
MX 853						T																													
MX 698									G			C															A			G	T		A	T	
NW 1212																																			
NW 2649																																			
NW 3363		G	G		C			C																											
NW 3715			C								T		A									G					G								
NW 3773																																			
NW 4040			C								T		A														G								
NW 4075			C								T		A														G								
NW 1285			C								T		A														G								
SA*1073															T		A										G								
SA*1415							A					C		A													A			G	T		A	T	
SA*1509							A					C		A													A			G	T		A	T	
SA 1105							A		G			C															A			G	T		A	T	
SA 1644							A					C		A													A			G	T		A	T	
SA 2157										T		A															G								
USA 9598			C								T		A														G								
USA 9162			C				A		G	G		C															A			G	T		A	T	
USA 1009			C		T		A		G	G		C															A			G	T		A	T	

Fig. 2. Genomic variation of HPV-31 isolates from Mexico, Norway, South Africa, and the United States of America in a 523-bp genomic segment, the 3′ half of the viral long control region. The variants are identified with the acronyms MX, NW, SA, and US according to the respective country of origin, and a number identifying the specific sample within the local collections.

Costa et al., 2002; Giannoudis et al., 2001; Kammer et al., 2000; Villa et al., 2000; Xi et al., 1997; Xi et al., 1998), and likely contribute to geographic disparities.

Some of us (I.E.C.M., R.O.L., A.R.M., J.F.G.G., and H.A.B.S.) are participating in a large ongoing epidemiolog-

ical study that aims to establish the relative prevalence of HPV types in women living in and around the city of Monterrey in the Mexican state of Nuevo Leon to identify high-risk HPV carriers for early intervention and to lay the foundation for future DNA diagnostic services and vaccina-
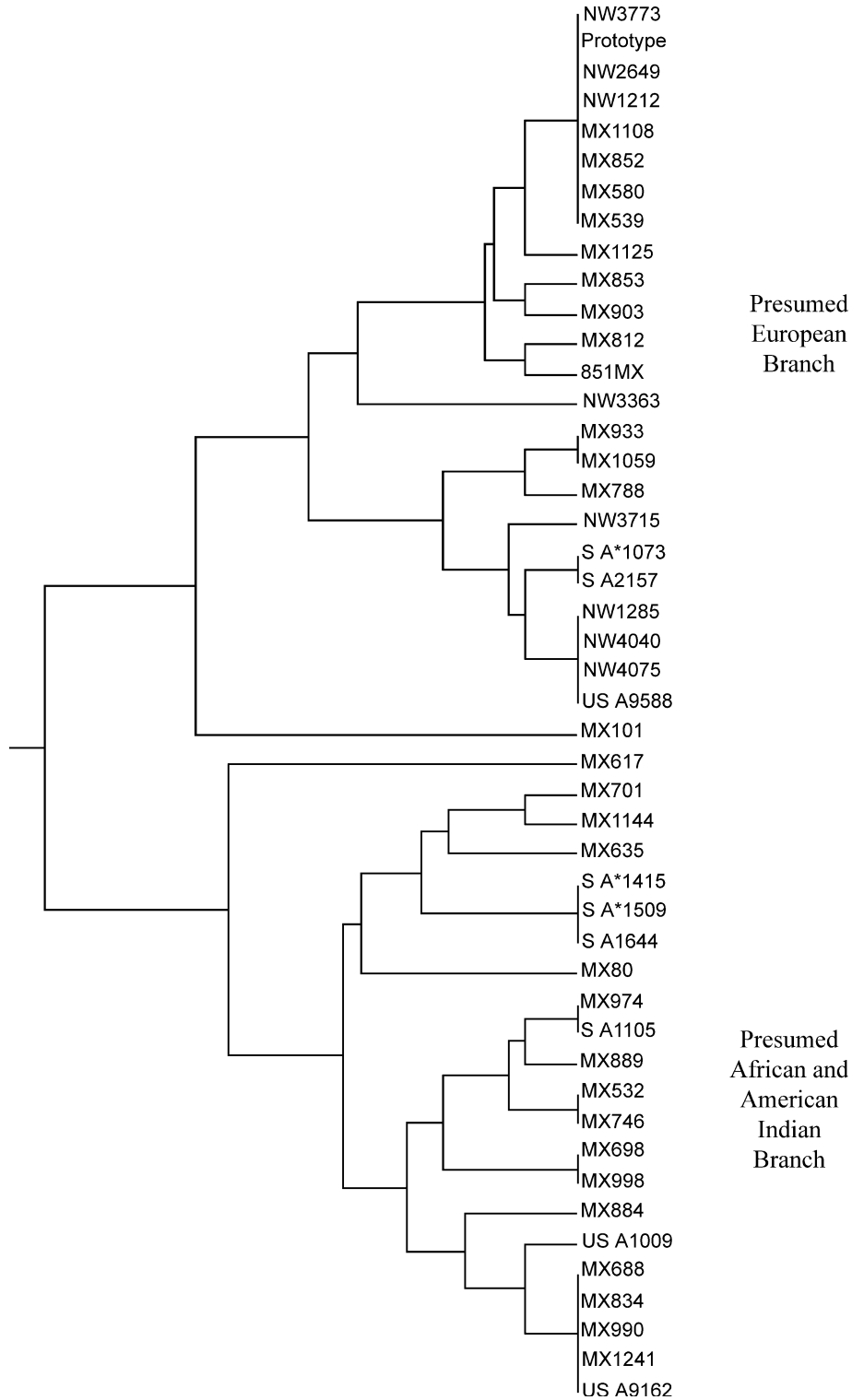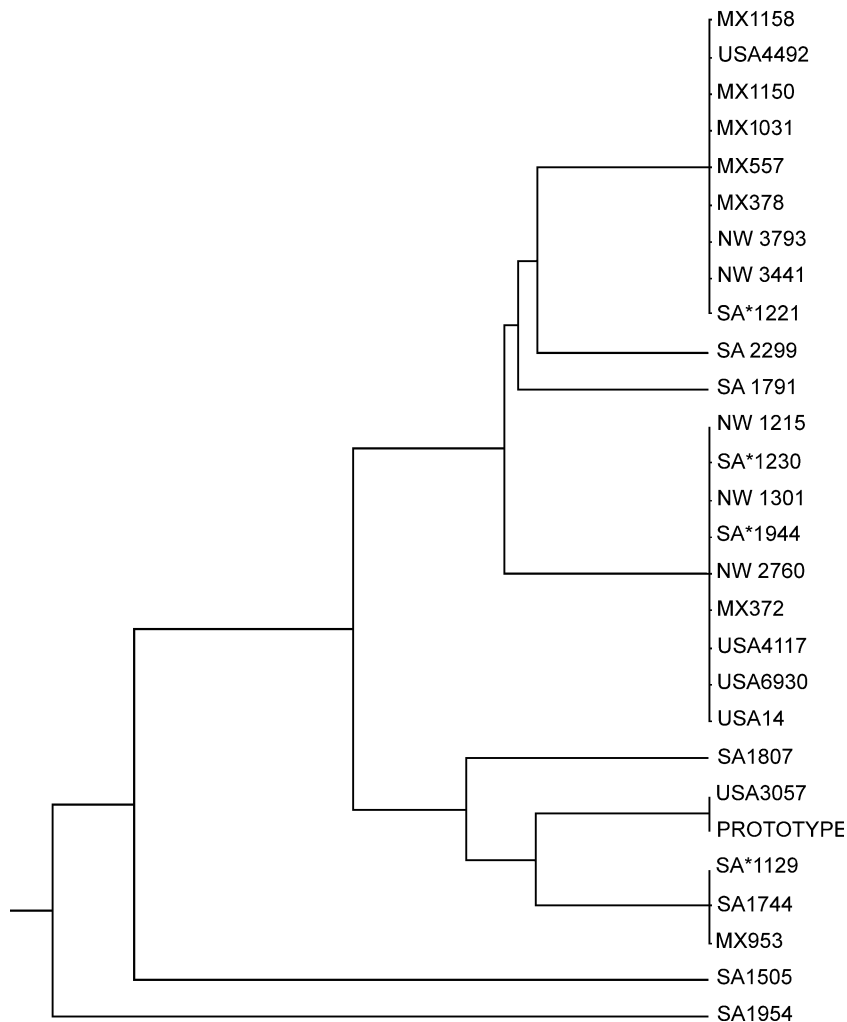
Fig. 3. Phylogenetic tree of HPV-31 variants from Mexico, Norway, South Africa, and the United States of America. The variants are identified with the acronyms MX, NW, SA, and US according to the respective country of origin, and a number identifying the specific sample within the local collections.

tion programs. This ongoing study will be published elsewhere and is not the subject of this paper. At the present state of this research, after investigating smears of 1200 patients, who were consecutively sampled without prior cytological diagnosis, we have identified HPV-16, 31, 18, and 35 as the most prevalent HPV types with 112, 29, 20, and 7 samples containing either of these types, respectively. It was the objective of the research reported here to determine the genomic variation of these four HPV types in this Mexican cohort to assign HPV-16 and HPV-18 variants to previously established intra-type phylogenetic trees, and to establish phylogenetic trees for the little studied types HPV-31 and HPV-35.

There is a strong evidence that evolutionary changes of HPV genomes occur at a very small pace, as it takes apparently several 10 000–100 000 years to generate about 1% sequence diversity (Chan et al., 1995; Ho et al., 1993; Ong et al., 1993). There is no evidence for recombination of HPV genomes that may confound a study of genomic variation. The variants of HPV types differ from one another by about 1% within genes and a few percent in some hypervariable regions such as the long control region (LCR) and part of the E2 gene (Hecht et al., 1995; Ho et al., 1993; Ong et al., 1993). One has to conclude that several hundred thousand years ago, when the human species evolved, all HPV types were already in existence with genomes very similar to those found today. Genetic distances among HPV isolates of one or a few percent evolved in parallel to the ethnic groups and the spread of humans around the earth. As a consequence, certain variants of HPV types predominated in defined and isolated ethnic groups, such as in the people who first colonized the American continent 12 000 years ago. In a country with diverse ethnic origins such as Mexico, where European immigrants mixed with native American Indians, today's population carries the HPV variants that were originally specific for either ethnic group. Variants of HPV types are best diagnosed by determining the sequence of their LCR. Biological and pathological differences between variants of any HPV type may originate from these particular differences of the LCR. More often, however, they may stem from functional differences among mutant proteins. due to the linkage between the corresponding genes and the LCR sequences, the latter are nevertheless sufficient for identification of variants with different biological properties.

Results

Most HPV-16 variants of the Mexican population are of American Indian origin

All HPV-16 isolates investigated so far belong to either of six phylogenetic branches of HPV-16 variants, namely two closely related African branches (Af1 and Af2), a European branch (E) closely related to an East Asian branch (As), and a branch with variants common in American Indians, which also occur in parts of Asia (AA) and which are closely related to variants specific for North American Indians (NA). The E variants are typified by the original HPV-16 reference clone, an isolate from a German patient,

	7256	7261	7270	7282	7394	7412	(insertion region)																		7413	7439	7535	7615	7719	7758
Reference	T	A	T	T	A	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	T	T	T	A
NW 3441							T	T	C	T	A	C	C	T	C	C	A	T	T	T	T	G	T	G	G	C				G
NW 3793							T	T	C	T	A	C	C	T	C	C	A	T	T	T	T	G	T	G	G	C				G
NW 1301							T	T	C	T	A	C	C	T	C	C	A	T	T	T	T	G	T	G	G	C			G	G
NW 1215							T	T	C	T	A	C	C	T	C	C	A	T	T	T	T	G	T	G	G	C			G	G
NW 2760							T	T	C	T	A	C	C	T	C	C	A	T	T	T	T	G	T	G	G	C			G	G
MX 378							T	T	C	T	A	C	C	T	C	C	A	T	T	T	T	G	T	G	G	C				G
MX 953							T	T	C	T	A	C	C	T	C	C	A	T	T	T	T	G	T	G	G	C				
MX 1150							T	T	C	T	A	C	C	T	C	C	A	T	T	T	T	G	T	G	G	C				G
MX 372							T	T	C	T	A	C	C	T	C	C	A	T	T	T	T	G	T	G	G	C			G	G
MX 1158							T	T	C	T	A	C	C	T	C	C	A	T	T	T	T	G	T	G	G	C				G
MX 557							T	T	C	T	A	C	C	T	C	C	A	T	T	T	T	G	T	G	G	C				G
MX 1031							T	T	C	T	A	C	C	T	C	C	A	T	T	T	T	G	T	G	G	C				G
SA*1129							T																		G	C				
SA*1221							T	T	C	T	A	C	C	T	C	C	A	T	T	T	T	G	T	G	G	C				G
SA*1230							T	T	C	T	A	C	C	T	C	C	A	T	T	T	T	G	T	G	G	C			G	G
SA*1944							T	T	C	T	A	C	C	T	C	C	A	T	T	T	T	G	T	G	G	C			G	G
SA 1505		C			C		T																		G	C				
SA 1744							T																		G	C				
SA 1791				A			T	T	C	T	A	C	C	T	C	C	A	T	T	T	T	G	T	G	G	C				G
SA 1807							T																		G	T	C			
SA 1954			C	A			T	T	C	T	A	C	C	T	C	C	A	T	T	T	T	G	T	G	G	C	G			G
SA 2299	G						T	T	C	T	A	C	C	T	C	C	A	T	T	T	T	G	T	G	G	C				G
USA 4492							T																		G	C				G
USA 4117							T	T	C	T	A	C	C	T	C	C	A	T	T	T	T	G	T	G	G	C			G	G
USA 6930							T	T	C	T	A	C	C	T	C	C	A	T	T	T	T	G	T	G	G	C			G	G
USA 3057							T																		G					
USA 14							T	T	C	T	A	C	C	T	C	C	A	T	T	T	T	G	T	G	G	C			G	G

Fig. 4. Genomic variation of HPV-35 isolates from Mexico, Norway, South Africa, and the United States of America in a 893-bp genomic segment, the 3′ half of the viral long control region.

and a variant differing in the diagnostic genomic fragment (genomic position 7450–7850) in a single position, 7521. AA variants are characterized by mutations in the positions 7485, 7489, 7669, 7764, and 7786. These genomic positions refer to the revised sequence of HPV-16. The position 7521 is identical to position 7519, and the other five mutations to 7483, 7487, 7667, 7762, and 7784 in the original phylogenetic classification (Ho et al., 1993).

We have detected in this Mexican cohort among 1200 smears 112 samples with HPV-16, 98 of these with variants from the AA branch, and 14 with European variants (Fig. 1).

European and African HPV-18 variants in a Mexican cohort

The HPV-18 reference clone is a Brazilian isolate and likely a representative of HPV-18 variants of American Indians. The LCR of European variants differs from this clone by mutations in the position 7529, 7567, 7592, and 7670, and African variants by additional 10 nucleotide changes in a 320-bp segment (Ong et al., 1993). Maximal differences between any two variants are 7.3%. Here, among

15 Mexican isolates, we found 13 belonging to the European cluster and 2 African variants (Fig. 1).

Mexican variants belong to both branches of a newly determined intra-type phylogenetic tree of HPV-31

HPV-31 was with 29 samples, the second most common HPV type in the Monterrey cohort. The genomic diversity of HPV-31 isolates has never been studied. Therefore, we amplified and sequenced from these isolates a 523-bp segment between the genomic positions 7527–137, which included the homologous part with the viral enhancer of the diagnostic 400-bp HPV-16 segment. In order to approach a hypothesis about a potential ethnic origin of the Mexican samples, we included in our analysis eight samples from European patients (Norway) and six samples from African patients or patients with mixed African ethnicity from South Africa.

In all isolates together, we detected nucleotide exchanges in 28 positions, and single nucleotide deletions in 7 additional positions (Fig. 2) in altogether 25 different variants. The

Fig. 5. Phylogenetic tree of HPV-35 variants from Mexico, Norway, South Africa, and the United States of America.

maximal distance between any two variants was 16 bp (3.1%), similar to that among HPV-16 variants (3.6%) (Fig. 2).

Phylogenetic trees, calculated either by the UPGMA or the NJ algorithm, placed all HPV-31 variants on two branches, one with all Norwegian variants and the original German reference clone. The other branch contained no European, but four of the six South African isolates. This asymmetric distribution suggests that the first branch lumps original European variants, the second one African variants. The first and second branch contained 13 and 16 Mexican and 1 and 2 US isolates, respectively (Fig. 3).

Several phylogenetic clusters of HPV-35 variants unite Mexican, European and African isolates

HPV-35 was, with seven samples, the forth most common HPV type in the cohort. The original HPV-35 clone has been isolated in the United States, and the ethnicity of the patient is unknown. The correct sequence of this HPV-35 isolate has been published under the abbreviation HPV-35h (see http://hpv-web.lanl.gov/stdgen/virus/hpv/compendium/htdocs/HTML_FILES/HPVcompintro4.html#comp94 in Myers et al., 1994), because a prior published sequence contained a large number of sequencing errors. Since only a single additional variant genome of this type has ever been reported (Stewart et al., 1996), we compared the Mexican isolates with 5, 10, and 5, respectively, HPV-35 samples from the same European, African, and US cohorts that had entered the HPV-31 study. For this comparison, we amplified an 893-bp genomic segment between positions 7146 and 187, and detected, in addition to the prototype, altogether 8 variants with a total of 10 nucleotide exchanges and a 16-bp insertion (Fig. 4). Maximal distances between any two variants were five mutations (0.6%). These HPV-35 variants formed several variant clusters rather than two- or three-branched trees as HPV-16, 18, and 31 (Fig. 5). All Mexican and Norwegian and three of the five US samples had a characteristic 16-bp insertion and differed within and between the two geographic cohorts by a single point mutation. Absence of the 16-bp insertion was specific for the reference clone and some of the South African isolates. We found another isolate of the reference clone a second time in the US, as well as another variant without the insertion.

Discussion

Our research shows that each of the four most prevalent HPV types exists in this Mexican cohort in form of numerous variants with significant genomic differences, which raises the possibility that these variants exhibit intra-type biological and pathogenic differences. Once DNA diagnosis or vaccination programs are considered for this population, it should be examined whether the diagnostic tools or epitopes are affected by mutations elsewhere in the genomes of these HPV-16, 18, 31, and 35 variants.

All variants of HPV-16 could be unequivocally assigned to either the European or the American Indian branch of the HPV-16 phylogenetic tree. HPV-16 is the only type for whom it is well confirmed by numerous epidemiological (Becker et al., 1994; Berumen et al., 2001; Da Costa et al., 2002; Franco et al., 1999; Giannoudis and Herrington, 2001; Giuliano et al., 1999; Xi et al., 1997, 1998) and molecular biological (Kammer et al., 2000; Villa et al., 2000) studies that genomic variation correlates with altered biological and epidemiological properties, which apparently result in increased carcinogenicity of African and AA variants. Except in American Indians and some Asian populations, AA variants are frequent in peoples with an American Indian component (Berumen et al., 2001; Lizano et al., 1997), but elsewhere, they were only found in Spain (Yamada et al., 1997), possibly because of reverse migration from Latin America.

The prevalence of 88% of the HPV-16 AA variant in this Mexican cohort is the highest ever detected in any part of the world. This observation and data from the papers cited above make it possible that exposure to the AA variants of HPV-16 contributes to the increased risk of Mexican women to develop cervical cancer. The high ratio of AA variants in Nuevo Leon is astonishing, as a large part of the population of this Mexican state is considered to be ethnically of Spanish origin, and even among individuals explicitly categorized as Mestizos, the admixture of Indian genes has been measured as maximally 40%, with 55% European and maximally 5% African genetic markers (Cerda-Flores et al., 2002). As HPV-16 infects the vast majority of all human populations, one might expect that the spread of the virus follows similar characteristics as the spread of host genes. It may be interesting to address the question of whether behavioral components explain this disparity, or whether HPV-16 AA variants spread more efficiently than E variants.

Alternatively, it may be that migration has significantly affected virus distribution in this region if groups that might once be kept apart by vast distances are mixing sexually. Population movements between Central American populations, Mexico, and Hispanic populations in the United States are well documented, and these migrations likely increase the chance of sexual contact between an émigré and men or women of the host culture. Ultimately, these social influences may heighten a Monterey woman's risk for acquiring viral variants that were historically absent or rare in this population.

The number of HPV-18 samples is too low for any conclusions. A predominance of European variants, as observed, would be expected, and the presence of African variants is a distinct possibility because of an ethnic African element in this population. The absence of the HPV-18 prototype from these samples is noteworthy, as this is a Brazilian isolate that had frequently been found again in South American cohorts (Ong et al., 1993).

We determined phylogenetic trees of HPV-31 and 35 variants based on LCR segments of these viruses that

overlapped with and extended the homologous segments of HPV-16 and HPV-18 that had been used for phylogenetic evaluations. HPV-31 variants are separated into two deep phylogenetic branches. It seems likely that the first branch with all European variants and 13 out of 29 Mexican isolates represents typical European HPV-31 genomes. The second branch with 4 of 6 African and 16 out of 29 Mexican isolates are difficult to assess. It seems reasonable to suggest that it may represent the original HPV-31 genomes of American Indians, because it would be difficult to explain a high prevalence of African isolates in a country with only a very small fraction of African immigrants. This phylogenetic similarity of African and American Indian variants would be somewhat reminiscent to the situation in HPV-16, where American Indian (AA) and African variants are more closely related to one another than to European variants.

HPV-35 variants fall into two categories, identified by the presence or absence of a 16-bp segment absent from the HPV-35 reference clone and four of nine African isolates. As this distinction has to be counted as a single mutation, this genomic difference as well as the generally low number of nucleotide differences does not allow establishing a stable tree for HPV-35 variants. Since all Mexican isolates are identical to some European or some African isolates, this cohort suggests that the HPV-35 type was either absent from America in pre-Columbian times or that American Indian HPV-35 variants were indistinguishable from those in Europe and Africa.

About 30% of all malignant tumors in women in Mexico are uterine cervix carcinomas (Gonzalez-Garay et al., 1992; Hernandez-Avila et al., 1998; Silva et al., 1999; Torroella-Kouri et al., 1998), making this cancer a leading oncological concern of public health. While this high prevalence likely will have some behavioral explanations and may also be caused by lack of appropriate medical care (Lazcano-Ponce et al., 1999a, 199b), our data raise the possibility that the specific viral load, that is, a unique epidemic with variants of HPV types that differ from those in Europe and the United States, contributes to this problem.

Materials and methods

Clinical specimens and DNA preparation

Among a cohort of 1200 consecutive women from several primary health care centers in Monterrey, Nuevo Leon, Mexico, a total of 161 specimens were positive for the four most common HPV types, namely 112 for HPV-16, 29 for HPV-31, 20 for HPV-18, and 7 for HPV-35 positive samples. All of these samples entered this study (designated as MX-Z, Z being the code number of the patients), except five samples with HPV-18 with insufficient DNA. These samples had been taken in the form of swabs during a large ongoing epidemiological study that will be published elsewhere by some of us (I.E.C.M., R.O.L., A.R.M., J.F.G.G., and H.A.B.S., in preparation). The 13 Norwegian swabs (NW-Z) were collected in Oslo, Norway, during gynecological consultations of these patients. Sixteen South Africa specimens (swabs) from Cape Town excluded white patients but were from two different ethnic groups that we refer to as black (SA*-Z) and mixed race (SA-Z) women. For all three cohorts, cytological diagnoses were done after obtaining these samples, and the clinical outcome did not influence the inclusion in this study. The samples from the United States of America were obtained in Los Angeles and derived from biopsies of anal neoplastic lesions in patients positive for infection with the human immunodeficiency virus. The use of these samples was approved by the Institutional Review Board of the University of California Irvine, and collection followed the respective patient protection rules of each of the four participating clinics in Monterrey, Oslo, Cape Town, and Los Angeles. Cervical DNA was extracted and purified following standard techniques. Briefly, the samples were digested with proteinase K, and the DNA was purified with phenol–chloroform. The specific HPV types were determined by PCR amplification with MY09/11 primers and sequencing as previously described (Bernard et al., 1994).

Table 1
Location and sequence of primers used in PCR amplification and sequencing reactions

LCR HPV type	Name	Location	Sequence (5′–3′)
HPV-16[a]	7478	7478–7477	GGGGTACCTCGGTTGCATGCTTTTTGGC
	7841	7861–7841	GGTCTAGACGGTTTGCACACACCCATGT
HPV-21	HPV31-8aF	7527–7550	AGTAGTTCTGCGGTTTTTGGTTTC
	HPV31-8aR	114–137	CCGAGGTCTTTCTGCAGGATTTTT
HPV-18[b]	7485	7465–7484	TCGGTTGCCTTTGGCTTATG
	7805	7806–7825	CGGTTGCATAAACTATGTAT
HPV-35	HPV35LCR-F	7146–7172	TATATTATGTGTTGTGGTGCCTGTTTG
	HPV35LCR-R	163–187	AATTTCATGGATGCTTTCTTCTACC
	HPV35LCRa-F	7418–7439	CGATTTCGGTTGCTGTTGGTAAG
	HPV35LCRa-R	11–33	CGTTTTCGGTCACTCCCTGTTTT

The HPV-16 and 18 primers have been introduced by Ho et al. (1991) and Ong et al. (1993).
[a] Primers published by Ho et al. (1991).
[b] Primers published by Ong et al. (1993).

PCR and DNA sequencing of LCR

A major segment of the LCR region was amplified with primer pairs specific for each HPV type. Table 1 lists sequences and locations of the primers. Reaction mixtures contained 20 mM Tris (pH 8.0), 100 mM KCl, 200 mM of each deoxynucleoside triphosphate, 2 mM MgCl$_2$, 10 mM each of the sense and antisense oligonucleotide primer, and 1 unit of Taq DNA polymerase (Promega, Madison, WI), although Go Taq DNA Polymerase (Promega) was used for HPV-16 amplification. Forty amplification cycles were run in the Eppendorff Master Cycler (Eppendorff, Hamburg, Germany) with a 94 °C denaturing step (30 s), a 60 °C annealing step (30 s), and a 72 °C extension step (1 min), including a denaturing step of 4 min and a final extension of 5 min.

PCR products were visualized by ethidium bromide agarose gel electrophoresis and purified by the QIAquick gel extraction kit (Qiagen, Hilden, Germany). The products were treated with 2 units of shrimp alkaline phosphatase and 10 units of exonuclease I (USB, Cleveland, OH) at 37 °C for 1 h to clean the reaction from primers and dNTPs. The enzymes were subsequently inactivated by heating at 72 °C for 20 min. The products were sequenced using the BigDye Terminator Cycle Sequencing kit (Perkin Elmer Applied Biosystems, CA). For each sample, two independent PCR products were generated and sequences from both orientations and internal primers (Table I) to exclude PCR artifacts. Sequence changes that were found at least twice in the same sample were counted as variants.

Sequence analysis

Sequence analysis was performed with ALIGN at the GENESTREAM network server as published by Pearson et al. (1997) to compare the HPV variant sequences with those of the reference sequences. In this study, the nucleotide positions of the HPV-18, HPV-31, and HPV-35 genomes were numbered according the reference sequences NC 001357, NC 001527, and X74477, respectively. HPV-16 sequence and base positions are numbered according of the last update of the HPV-16 reference genome sequence (Los Alamos National Laboratory, http://hpv-web.lanl.gov/stdgen/virus/hpv/compendium/htdocs/). The HPV-35 sequence reference used in this study was HPV-35H in the same database, because of multiple errors in the original HPV-35 sequence.

Phylogenetic analysis

HPV-16 and HPV-18 variants were compared with those forming published phylogenetic branches (Ho et al., 1991, 1993; Ong et al., 1993; Stewart et al., 1996). Phylogenetic analyses of HPV-31 and HPV-35 variants were performed using MEGA version 2.1 as published by Kumar and Gadagkar (2001). The phylogenetic trees were determined using the unweighted pair-group method with arithmetic average (UPGMA) and Neighbor joining (NJ) method.

Nucleotide-sequence accession numbers

The nucleotide sequences of all new HPV-31 and HPV-35 variants have been entered into GenBank with the accession numbers AY453865–AY454064.

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