RESUMEN AUTOBIOGRÁFICO

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Candidato para el Grado de

Doctor en Ciencias con Especialidad en Biología Molecular e Ingeniería Genética.

Tesis: **EVALUACIÓN DE LA VARIABILIDAD GENÉTICA Y EPIGENÉTICA DEL HPV EN UNA POBLACIÓN DEL NORESTE DE MÉXICO.**

Campo de estudio: Medicina Molecular

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Virology 319 (2004) 315–323
VIROLOGY
www.elsevier.com/locate/yviro

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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Received 2 September 2003; returned to author for revision 4 November 2003; accepted 7 November 2003

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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Keywords: Genomic diversity; Papillomavirus; Population

Introduction

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

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doi:10.1016/j.virol.2003.11.009

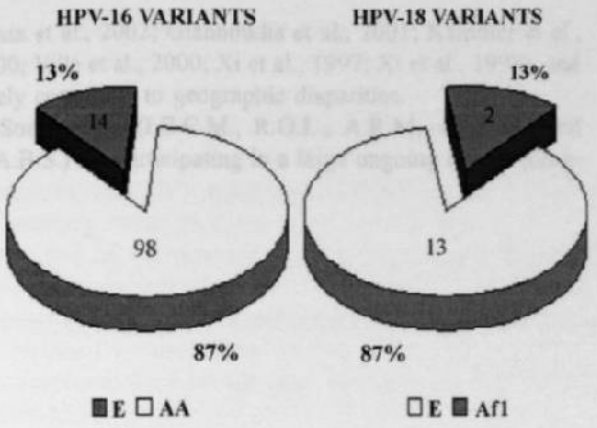

Fig. 1. Prevalence of HPV-16 and HPV-18 variants in a cohort from Monterrey, Nuevo Leon, Mexico.

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

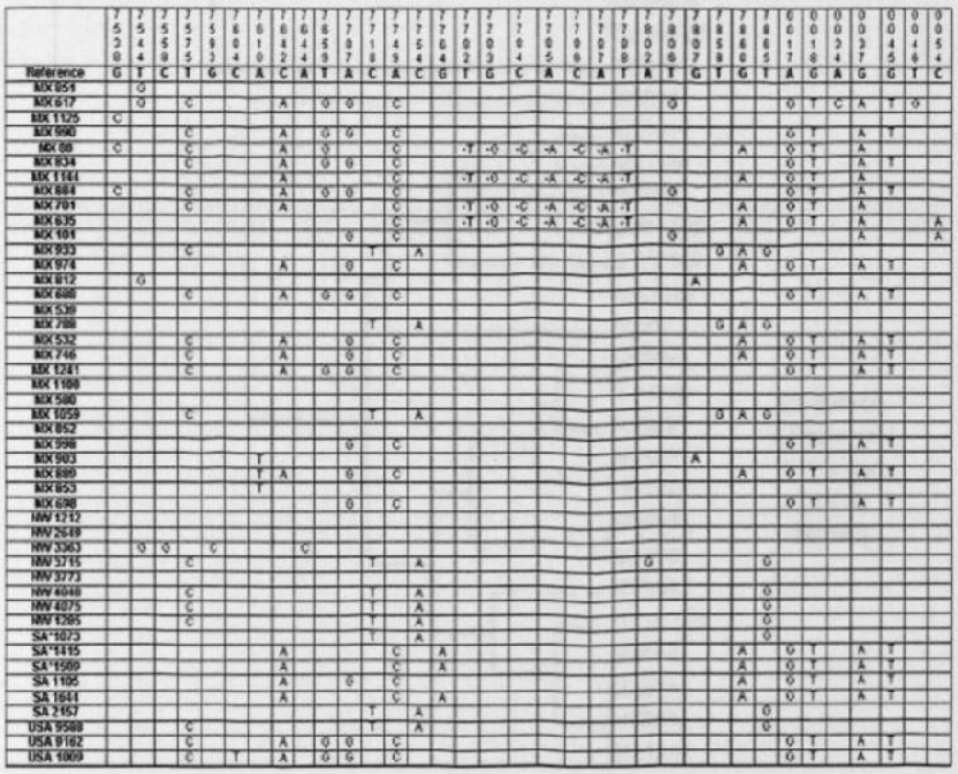

Fig. 2. Genomic variation of HPV-31 isolates from Mexico, Norway, South Africa, and the United States of America in a 523-bp genomic segment, the 3′half of the viral long control region. The variants are identified with the acronyms MX, NW, SA, and US according to the respective country of origin, and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

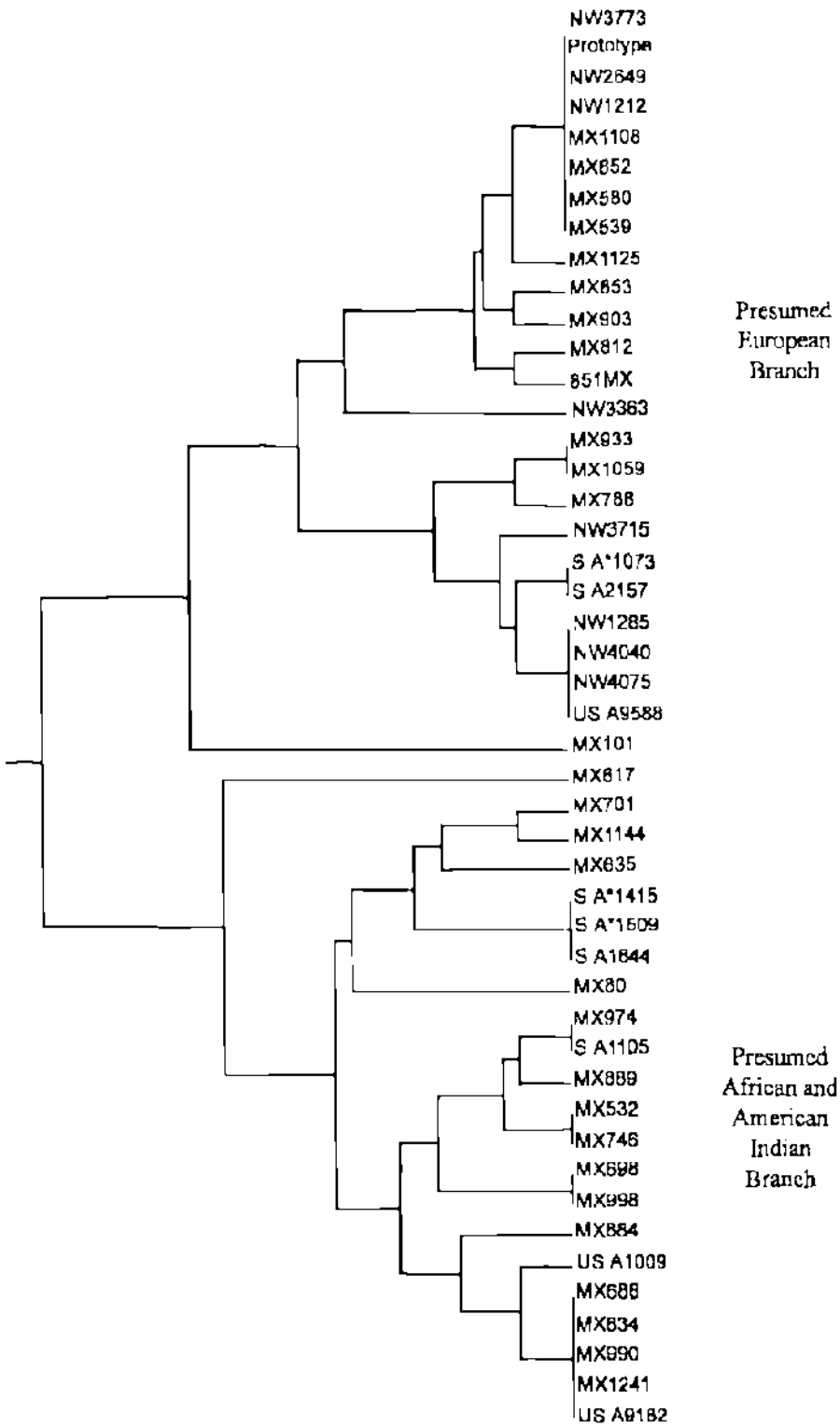

Fig. 3. Phylogenetic tree of HPV-31 variants from Mexico, Norway, South Africa, and the United States of America. The variants are identified with the acronyms MX, NW, SA, and US according to the respective country of origin, and a number identifying the specific sample within the local collections.

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,

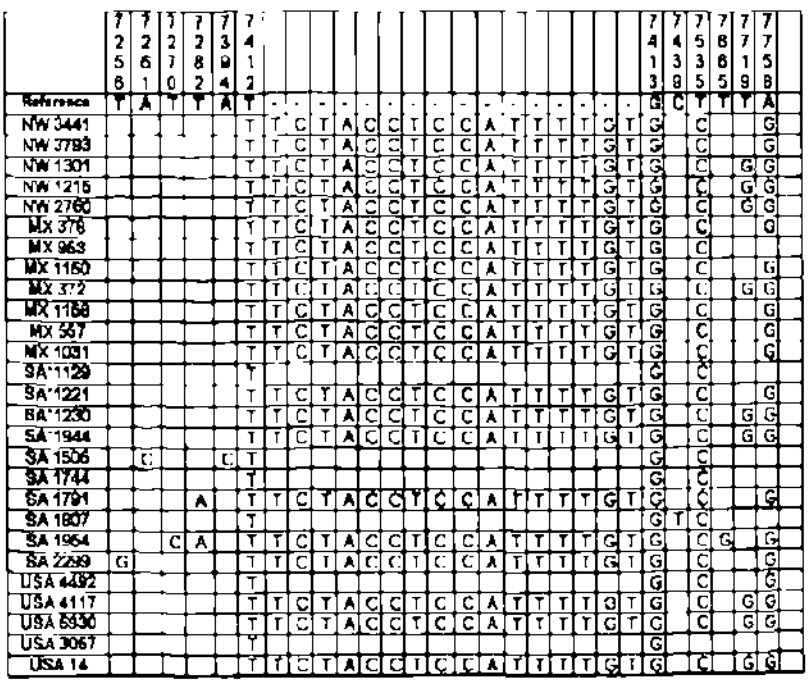

Fig. 4. Genomic variation of HPV-35 isolates from Mexico, Norway, South Africa, and the United States of America in a 893-bp genomic segment, the 3′half of the viral long control region.

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 7570, 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

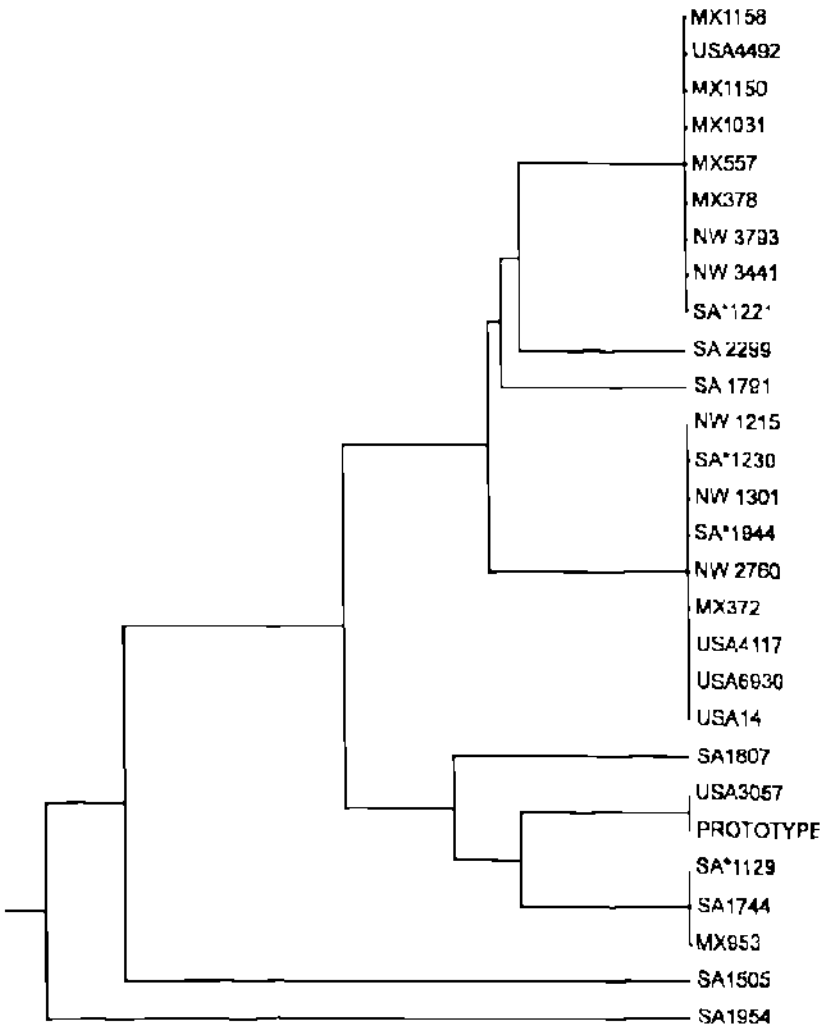

Fig. 5. Phylogenetic tree of HPV-35 variants from Mexico, Norway, South Africa, and the United States of America.

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 emigre 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; Tornella-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 1) 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.

Acknowledgments

We are grateful to Drs Maria E. Diaz-Garcia, Patricia Lopez-Reyes, and Cesar H. Rosas-Herrera for support of this study, and Bruce R. Allan for typing the samples from Cape Town. We also acknowledge Roche Molecular Systems for provision of reagents for typing HPVs in the Cape Town samples. This research was funded by a Collaborative Research Grant from UC MEXUS-CONACYT to I.C., L.V., H.A.B.S, and H.U.B., and by funding of the work of H.U.B. by the Cancer Research Institute of the University of California Irvine, and NIH grant ROI CA-91964 to H.U.B.

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JVI 01281-04, version 2

Conserved Methylation Patterns of Human Papillomavirus-16 DNA in Asymptomatic Infection and Cervical Neoplasia.

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Running title: HPV-16 DNA methylation during cervical infections.

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ABSTRACT.

DNA methylation contributes to the chromatin conformation that represses transcription of the human papillomavirus-16, which is prevalent in the etiology of cervical carcinoma. In an effort to clarify the role of this phenomenon in the regulation and carcinogenicity of HPV-16, 115 clinical samples were studied to establish the methylation patterns of the 19 CpG dinucleotides within the long control region and part of the L1 gene by bisulfite modification, PCR amplification, DNA cloning and sequencing. We observed major heterogeneities between clones from different samples as well as between clones from individual samples. The methylation frequency of CpGs was measured at 14.5%. In addition, 0.21 and 0.23%, respectively, of the CpA, and CpT sites, indicators of de novo methylation, were methylated. Methylation frequencies exceeded 30% in the CpGs overlapping with the L1 gene and were about 10% for most other positions. A CpG site located in the linker between two nucleosomes positioned over the enhancer and promoter of HPV-16 had minimal methylation. This regions forms part of the HPV replication origin, and is close to binding sites of master-regulators of transcription during epithelial differentiation. Methylation of most sites was highest in carcinomas, possibly due to tandem repetition and chromosomal integration of HPV-16 DNA. Methylation was lowest in dysplasia, likely reflecting the transcriptional activity in these infections. Our data document the efficient targeting of HPV genomes by the epithelial methylation machinery, possibly as a cellular defense mechanism, and suggest involvement of methylation in HPV oncogene expression and the early-late switch.

INTRODUCTION.

The spread of human papillomaviruses (HPV) into mucosal and cutaneous epithelia can result either in a clinically asymptomatic infection not associated with pathological changes or the development of neoplastic disease. While the host immune system likely plays a role in the elimination of the virus, it is unclear which factors determine whether an infection leads to long-term persistence in the absence of symptoms or to neoplastic

progression. The understanding of these alternative outcomes is important in the case of the 18 "high-risk HPV types" that are most frequently associated with cervical carcinomas. Population-based data suggest that "high risk" HPV infections occur frequently relative to the number of carcinomas that result. Cervical cancer cases generally number in fewer than hundreds of cases per 100,000 women, while the incidence rate of becoming infected at least once during the life is believed to significantly exceed 50% (6,35,46,49). Further research is needed to discriminate whether there are two different stages of infection, namely asymptomatic productive infection and latency. In the latter case the virus would be maintained not only without changes to the host cell, but also without production of viral particles. While the former may represent immunological delays or failures, the latter may serve as a model for malignancies that evolved late in life.

The regulation of HPV oncogene transcription as a major modulator of productive HPV infections is a focus of debate. It has been reported that HPV oncogene transcription is only active in differentiating epithelial cells, but is repressed in undifferentiated cells by the factors CDP and YY1, which alter, in collaboration with histone deacetylases (HDACs) the chromatin conformation in ways unfavorable to transcription (1,26,39). This mechanism was identified in vitro by transcriptional studies and is supported by immunocytochemical data of HPV infected tissue in situ (37). A second example are data that glucocorticoids and progesterones increase HPV oncogene transcription (11,29). These molecular mechanisms may explain the increased risk of cervical carcinogenesis by multiparity, use of antiovulants (35), and possibly stress. Yet another mechanism is based on the observation that transcriptional stimulation results from the insertion of HPV genomes into cellular chromosomes during cancer progression. This event interrupts a negative feedback loop (36,43) and stimulates transcription depending upon nuclear matrix attachment regions (38).

DNA methylation provides an additional means to regulate HPV transcription since the repression of cellular and viral gene expression can occur by the introduction of methyl groups to cytosine residues (5,14,19,20,27,31,48). Methylated cytosines are maintained

in a stable form when they occur in the palindrome CpG (meCpG) (23). meCpGs can result in displacement of transcription factors (44), but more often lead to changes of the chromatin configuration under the influence of histone deacetylases (HDACs) (21). This mechanism resembles the modifications triggered by complexes between HDACs and the transcription factors CDP and YY1, since HDACs can either form complexes directly with the DNA methylases or with the repressors of the MeCP2 family, which recognize and bind meCpGs (5,8,21).

Previous reports more than twenty years ago on HPV-1 and the cottontail rabbit papillomavirus suggested that papillomaviruses are targeted by cellular DNA methylation, although limited analytical power, namely methylation sensitive restriction enzymes, was accepted as the state of the art of that time (9,13,40,47). Research on the methylation of HPVs was extended recently to the high-risk HPV type HPV-16 (2,22) by bisulfite modification and DNA sequencing. Ongoing pilot studies confirmed the occurrence of DNA methylation in HPV-6, 11, 18, and 31 (1a, and our unpublished observations). The consequence of CpG methylation is repression of HPV transcription as shown by transfection of in vitro methylated HPV DNA (34) and by in vivo studies with the cell lines CaSki and SiHa (2,45). Our research concentrates presently on HPV-16, the most prevalent HPV type in cervical carcinoma (6). The observations cited above provide evidence for three mechanisms, which may occur concomitantly, namely for methylation (i) of HPV-16 genomes in episomal form in cells in asymptomatic smears, (ii) of HPV-16 genomes that form tandem arrays in cancer lesions, and (iii) for de novo methylation in a somatic tissue, namely mucosal epithelium.

The goal of the present study was to further investigate the methylation patterns of the CpG dinucleotides contained within the long control region and L1 region of the HPV-16 genome in a large collection of clinical samples of the human cervix. We wanted to determine whether a thoroughly studied segment of the HPV-16 genome follows defined methylation patterns and whether certain CpG sites are targeted more or less frequently depending upon the state of the cervical infection. In addition, we wanted to estimate the amount of de novo methylation in an HPV infected cell population by measuring

methylation in other dinucleotides besides CpGs, namely CpAs, CpTs, and CpCs. Each of these issues were addressed by the sequencing of multiple HPV-16 genomes from individual samples to further characterize the hetero- or homogeneity of methylation within each lesion while also addressing the relationship between the observed patterns as they relate to the presence or absence of clinical disease.

MATERIALS AND METHODS.

Origin of samples. CaSki cells had served for two decades as a paradigm of a cervical carcinoma derived cell line with 500 chromosomally recombined HPV-16 genomes. All clinical samples were archival. The carcinoma samples were fresh frozen, stored at –70°C and histologically confirmed before and after DNA extraction. The samples were obtained from three sources, namely from patients examined and treated by two of us (B.H. and K.I.) in Oslo, Norway (21 from asymptomatic patients, 17 from CIN I to III grade lesions, 29 from squamous carcinomas), from patients examined during regular gynecological diagnosis in several different hospitals in Monterrey, Mexico, as published by us recently (10) (30 from asymptomatic patients, 13 from cervical carcinomas), and from the Cancer Center of the University of California Irvine (five cervical carcinomas).

DNA preparations. All DNA preparations were done with Qiagen DNA purification kits to assure removal of protein contaminations.

Bisulfite modifications. For bisulfite treatment (16), 50-1000 ng sample DNA supplemented with one µg of salmon sperm DNA in a total volume of 18 µl in water were denatured with 2 µl of 3M NaOH and incubated at 37°C. After denaturing, 278 µl of 4.8M sodium bisulfite and 2 µl 100 mM hydroquinone were added and incubated in a thermal cycler for 20 cycles each at 55°C for 15 minutes and 95 °C for 30 seconds. The modified DNA was desalted with the QIAquick PCR purification protocol. The modified DNA was desulfonated by adding 5.5 µl of 3M NaOH, 5µg glycogen and incubation at 37°C for 15 minutes. The DNA was precipitated with 5.6 µl of sodium acetate and 150 µl

of 100% ethanol. The pellet was washed with 70% ethanol and dissolved in 30-50 µl TE buffer (10mM Tris-HCl pH 8, 1 mM EDTA).

Polymerase Chain Reactions, Primers, T/A cloning and DNA sequencing. The modified DNA was amplified in form of three amplicons: Part of the L1 gene and the 5'LCR with the primers 16msp3F (position 7049-7078, AAGTAGGATTGAAGGTTAAATTAAAATTTA) and 16msp3r (position 7590-7560, AACAAACAATACAAATCAAAAAAACAAAAA); the HPV-16 enhancer with the primers 16msp4F (position 7465-7493, TATGTTTTTTGGTATAAAATGTGTTTTT) and 16msp7R (position 7732-7703, TAAATTAATTAAAACAAACCAAAAATATAT); and the HPV-16 promoter with the primers 16msp5F (position 7748-7777, TAAGGTTTAAATTTTTAAGGTTAATTAAAT) and 16msp8R (position 115-86, ATCCTAAAACATTACAATTCTCTTTTAATA). The sequences of the primers were designed according to the genomic sequence of HPV-16 (24) assuming conversion of all cytosine residues into uracils. PCR was carried out in a 25 µl volume containing 0.2 mM of each of the four dNTPs, 10pmol of the primers, 2 mM $MgCl_2$ and 1 unit of Ampli Taq Gold (Perkin-Elmer). The PCR started at 94°C for 1 min, followed by 40 amplification cycles (denaturing at 94 °C for 10 sec, annealing at 58 °C for 30 sec and extension at 68 °C for 1min) with final extension at 68 °C for 7 min. The presence of PCR products was verified by agarose gel electrophoresis, and confirmed amplicons were cloned with the TOPO TA cloning kit for sequencing (Invitrogen). Cloned DNAs were sequenced by Big Dye terminator v3.1 Cycle Sequencing (Applied Biosystems).

Statistical analysis. Descriptive and tabular statistics were used to explore these data. Graphical representations of the CpG methylation frequency were estimated from the data. For some sites, DNA could not be amplified and data were treated as missing for these analysis. The frequency of methylation of each CpG, CpA, and CpT site was compared among three diagnostic outcome groups: squamous cell carcinoma, cervical intraepithelial neoplasia (CIN), and specimens from asymptomatically infected women (no identifiable lesion, NIL). The Fisher's Exact Test was used to test the hypotheses that the methylation frequencies for cancers and CINs were each greater than the methylation

frequency of specimens from asymptomatically infected women. Additionally, the Student's t Test was used to test the hypotheses that the observed methylation frequency at each site in each diagnostic outcome category was statistically significantly greater than zero. A total of ninety-five statistical comparisons were performed and we adjusted the level of significance for our statistical tests by using Bonferroni's adjustment for multiple comparisons. The frequency of methylation on the many CpA and CpT sites examined was small; thus, *p*-values less than 0.05 are reported, even though they may not meet the most stringent test limits of multiple comparisons.

RESULTS.

Study design. We approached this study intending to expand upon the manner in which we previously had investigated CpG methylation of HPV-16 DNA. Our original observations were based upon bisulfite modified DNA from cell lines and clinical samples, which were then amplified with primers specific for the DNA in which the cytosine residues had been converted to uracil groups, followed by direct sequencing of the amplification product (2). At the beginning of our new study, we made observations with DNA from CaSki cells as well as with clinical samples (data not shown) that sequencing reactions frequently showed overlapping C and T signals, which could not be suppressed by changing the temperature or the length of the bisulfite reaction. We concluded that cell populations from the same source contained mixtures of HPV-16 genomes with CpGs or meCpGs in the same position. Therefore, we altered our protocol, cloned the amplicons in E. coli plasmids and randomly selected for sequencing five independent E. coli colonies derived from each sample. This was done in order to identify divergently methylated HPV-16 genomes assuming there may be similar amounts of methylated and unmethylated molecules. We acknowledge that it is impossible to analyze mixtures of methylated and unmethylated molecules in samples where one DNA type vastly outnumbers the other one.

The HPV-16 genome is a circular double-stranded DNA with a size of 7906 bp. An 850 bp segment between the 3' end of the L1 gene and the 5' end of the E6 oncogene is called the long control region (LCR), since it contains most of the cis-responsive elements that regulate transcription, which occurs unidirectionally toward the E6 gene (3). The principal transcriptional start site is called p97 or E6 promoter and is located five bp upstream of the ATG of the E6 gene. We decided to standardize the analysis by amplifying and sequencing a genomic segment between the positions 7079 and 85, which includes 19 CpGs. Three of these 19 CpGs were derived from the 3' end of the L1 gene (7091, 7136, and 7145), five from the 5'-segment of the LCR (7270 to 7461), which makes yet incompletely understood contributions to the transcriptional process (39,42), six from the transcriptional enhancer (7535 to 7862), and five from the E6 promoter (31-58). We selected this segment in order to detect possible correlations of methylation targets with known functional units of the HPV-16 genome.

It is well known that bisulfite modification does not only alter cytosine residues, but introduces nicks in DNA. As a consequence, it is impossible to PCR amplify with high efficiency large genomic segments with sizes exceeding a few hundred base pairs. Because of this limitation, we had to dissect the 913 bp region that we wished to analyze into three amplicons, spanning between the positions 7091 to 7461, 7535 to 7695, and 7862 to 58. In other words, while we aimed to gather methylation information from five HPV-16 DNA molecules from each sample, data for each of these three segments represent the methylation status of different, non-contiguous molecules.

During the analysis of clinical samples, we regularly included as negative and positive controls DNA from SiHa and CaSki cells. The only HPV-16 genome in SiHa has no meCpGs throughout the enhancer and promoter region. Previously published findings based on direct sequencing of the amplification product showed that most of the 500 endogenous HPV-16 genomes in CaSki cells appeared to be completely methylated throughout this region (2).

In order to standardize the analysis of the clinical samples in this study, we aimed to analyze five bisulfite modified, PCR amplified and bacterially cloned HPV-16 segments: 51 asymptomatic infections (NIL); 17 low and high grade cervical intraepithelial neoplasia (CIN); and 47 from cervical carcinomas for each of the 19 CpGs in the L1-LCR segment described above. This strategy aimed to give information about a total of 10,925 CpGs. Unfortunately, some of our samples did not contain sufficient DNA to complete this study, and in these cases (51 out of 115 samples) we had to eliminate the analysis of the L1 and 5'-LCR segment. As a result, our study reports the methylation status of only 8,885 CpG residues: 6,080 representing information about all 19 CpG residues in 320 clones, and 2,805 the methylation status of 11 CpGs in 255 clones from the enhancer and promoter.

It was not possible to confirm the physical state of HPV genomes in this study. It is generally accepted, however, that HPV genomes are episomal in latent infection and early dysplasia (CIN I), two of the pathological groupings we studied, while they are most often chromosomally integrated in carcinomas (12).

Heterogeneous DNA methylation patterns in the HPV-16 genomes of the CaSki cell line. In a previous analysis (2) of HPV-16 DNA in CaSki cells involving one of us, amplicons were directly sequenced after bisulfite modification and PCR amplification, and the data obtained with this strategy suggested that all CpGs overlapping with the enhancer and promoter (positions 7535 to 58) were completely methylated in most viral copies (2). Here we report a re-analysis based on sequences of individual clones of modified and PCR amplified DNA, which also extended the analyzed segment in the 5' direction. In contrast to the published data, Fig. 1 shows that none out of 15 clones was completely methylated, but each clone contained between one and five (out of 19) unmethylated CpGs. None of five clones was methylated in position 7270 (which had not been previously analyzed), and methylation was infrequent at the positions 7535, 7554 and 7862. Based upon these findings, we conclude that the 500 HPV-16 genomes of CaSki cells are not homogenously methylated, but that there are differences either among tandemly repeated intrachromosomal copies or between copies inserted in different

chromosomal locations (45). We reexamined this assumption by directly sequencing the bisulfite modified and PCR amplified products from several independent analyses of CaSki DNA. In these experiments we observed in all sequence readouts overlapping C and T peaks at the positions 7270, 7535, 7554, and 7862, with the C peak slightly exceeding the T peak. This confirms the heterogeneous methylation of HPV-16 molecules in CaSki and the fortuitous selection of an excess of unmethylated molecules in Fig. 1.

Different CpGs are altered with different frequencies. Fig. 2 depicts the methylation/lack of methylation for each of the 8,885 CpG sites evaluated in this study. Altogether, we found 1,292 methylated CpGs, i.e. 14.5% of all CpGs were methylated. Fig. 3 shows the relative frequency of methylation of the 19 sites. Approximately half (51 out of 115) of the specimens could not be amplified between 7091 and 7461. Thus, for this region, the reduced size of our sample may have limited our power to detect statistically significant relationships across sites and across diagnostic outcome groups. Nonetheless, these data point to systematic differences of probabilities of each site to become methylated. For example, 29 to 41% of the CpGs at the positions 7091, 7136, and 7145 were methylated (the only CpGs within a gene, the late gene, L1), while methylation was only around 10% for most other sites. There are three regions where methylation frequencies reached minima, namely the positions 7270, 7554, and, most pronounced, with a frequency of only 3%, position 7862. Remarkably, these three regions coincide with the same three unmethylated positions detected in the LCR of HPV-16 genomes in CaSki cells.

For a subset of these samples, we performed control experiments and directly sequenced the amplification product after bisulfite sequencing. All of these controls confirmed that the individual DNA molecules that were sequenced after plasmid cloning correlated with the ratios that existed in situ: Whenever we found hypomethylated or unmethylated clones, we also observed hypomethylation by direct sequencing, and hypermethylated clones correlated with highly methylated directly sequenced DNA. While it is obvious

that five samples represent a complex mixture of molecules with a low statistical power, they are nevertheless a good representation of the diversity in situ.

Correlation of CpG DNA methylation patterns with pathology of the infected cell population. The bar diagram shown in Fig. 4 separates the methylation frequencies in Fig. 3 by diagnostic outcome and visualizes CpG methylation in relation to the pathological status of the HPV-16 infection, i.e. asymptomatic infections (NIL, grey bars), CIN lesions (black), and carcinomas (white). For cancer specimens, the proportion that showed methylation of CpGs was 4% at 7862 and ranged between 12% and 53% for the remaining CpG sites (white bars). The proportion of all CpGs that were methylated was significantly greater than zero (i.e., for CpG sites 7091–7695 and 31-58: using Student's t Test, $p<0.0001$). An exception was position 7862, where methylation occurred between 2 and 4% of test specimens, and we found no difference between CINs and NILs nor carcinomas and NILs (i.e. using Fischer's Exact Test, $p=1.0$ and $p=0.6$, respectively). CIN lesions (black bars) showed the lowest methylation frequency of the specimens tested, and methylation frequencies were more often intermediate in asymptomatic patients (grey bars). For example, for CIN specimens, we were able to detect methylation frequently at position 7145 (i.e. 33%, using Student's t Test, $p<0.0001$). At all other sites, the frequency ranged from 0 to 17% but none proved to be statistically significant. However, for specimens acquired from asymptomatically infected women, methylation frequencies greater than zero were detected at most CpGs (i.e. positions 7091, 7136, 7428, 7434, 7535 to 7695 and 31-43 and position 58, ranging from 6% at 37 to 33% at 7091, using Student's t Tests, $p<0.0001$).

For carcinomas, 42% of specimens showed methylation at CpG sites 7136 and 7145. In comparison, only 13% and 9% of sites at 7136 and 7145 were methylated in specimens from asymptomatically infected women (i.e., for each comparison, using Fisher's Exact Test, $p<0.00002$). Additionally, at site 7145, 33% of CIN specimens showed methylation compared to 9% of specimens from asymptomatically infected women (similarly, $p=0.0005$). Last, when compared to specimens from asymptomatically infected women, methylation in cancer samples was significantly elevated at CpG positions 31, 37, 52 and

58 (using Fisher's Exact Test, for 31: 17% vs 6%, $p<0.0004$; for 37: 17% vs 6%, for 52: 18% vs 4% and for 58: 18% vs 7%, and for each comparison, $p<0.0001$; for 43: 17% vs 7%, $p<0.002$).

The minima at the positions 7270 and 7535/7554 are pronounced in all three groups, while the minimum at position 7862 is only clear for carcinomas and asymptomatic patients. We found no statistically significant difference in the methylation frequencies for these sites between carcinomas, CINs, and specimens from asymptomatically infected women using Fisher's Exact Test. Methylation at 7862 is also low in the case of CIN lesions, but it does not form a dip, as there is a nearly complete lack of methylation in all promoter positions 3' of 7862, and we found no statistically greater frequency of methylation at this site when pathology groups were compared. Thus, in nearly all positions, methylation is highest in carcinoma lesions (white bars) and lowest among CIN lesions (black bars).

MeCpA and meCpT dinucleotides identify de novo methylation of HPV-16 DNA in cervical epithelia. In the methylated state of the palindromic sequence CpG the cytosine residues of the upper and the lower strand are normally both methylated. The two replication products of a meCpG containing DNA are hemimethylated, and methylation of the unmethylated cytosine is restored by the maintenance DNA methylase DNMT1. Toward de novo methylation (44a, 47a), DNMT3a and DNMT3b are considered responsible to target unmethylated cytosine residues, while their contributions to maintenance methylation have recently been demonstrated (5,14,20). While this occurs preferentially at CpGs, changes at CpA and CpT dinucleotides can occur as well, although with less efficiency than CpGs (14,30). CpC methylation is known to be very rare. The methylation state of meCpAs and meCpTs is not maintained during replication, as one of the replication products does not carry a record of the methylation, and the other strand is becoming diluted in multiple rounds of replication. As a consequence, the detection of meCpA and meCpT residues is proof that the sequenced DNA molecule or one of the immediate precursors had become methylated, and it does not constitute a record of a long term maintenance of methylation. A record of these three methylated

dinucleotides in HPV-16 genomes therefore amounts to a documentation of active de novo methylation in the cell populations carrying these HPV-16 genomes.

Toward measuring CpA, CpT and CpC methylation, we investigated the same nucleotide sequence output of all 115 samples in quintuplets, that had led to the data in Fig. 2. We detected 58 meCpAs and 49 meCpTs residues, among a total of 26,785 and 20,845, respectively, and no meCpC dinucleotide at all. We also found six additional meCpGs in CpG positions diverging from the HPV-16 reference sequence, apparently genomic variants of HPV-16 (10). The total number of meCpAs and meCpTs in the 115 samples is incomplete, however, since the three amplicons, originally designed only to detect CpGs, do not overlap in genomic regions that did not contain CpGs and were therefore excluded from the original strategy.

Fig. 5 maps the distribution of meCpAs and meCpTs within the three amplicons across the L1-LCR segment. The numbers along the x-axis of this figure indicate the genomic positions (24) of cytosine residues that are part of a CpA or CpT dinucleotide, while the y-axis represents the frequency of finding a methylated C at this position. The raw data that led to this figure can be obtained upon request, as a representation in form of a figure or a table would be unwieldy and uninformative to most readers. Inspection of Fig. 5 suggests clusters of these two methylated dinucleotides between the positions 7268-7327 and 7840-24. These two regions overlap with minima of CpG methylation and may suggest that de novo methylation is more efficient in nucleosomal linkers than in the nucleosomes. Our analyses suggested methylation was detectable frequently at positions 7268 and 7317, and less frequently at positions 7840, 7857, 7876, 7882, 7886, 7903, 2, 13, 73, and 77 (i.e., testing the hypothesis that the mean frequency of cytosine methylation was greater than the null, using Student's t-test, p=0.0015, p=0.0045, p=0.0015, p=0.0046, p=0.0015, p=0.0009, p<0.0001, p=0.0009, p=0.0046, p=0.0003, p=0.0026 and p=0.0046, respectively). Also, statistically we detected less precisely cytosine methylation at dinucleotide positions 7301, 7327, 7554, 7602, 7689, 7829, 7829, 7841 to 7845, 7874, and at position 5 (i.e., p=0.01 for each). Thus, using the most

stringent level of significance, our analyses detected methylation at the maxima at positions 7886 and at position 13.

Although sparse, when data were stratified by pathology outcomes, our analyses suggested that methylation at particular positions was detectable with statistical significance. For women with CIN, CpA was methylated at position 7268 (i.e., p=0.0038). For women evidencing NIL, these analyses suggested that CpT was methylated at position 7840 and CpAs were methylated at positions 7857, 7876, 7882 and 7886 (i.e., p=0.014, p=0.014, p=0.0079, p=0.014, p=0.0014, respectively). For women with carcinomas, we were unable to precisely detect nucleotide regions where cytosines were methylated on CpA and CpT.

DISCUSSION.

HPV-16 CpG methylation: preferred and protected sites. We have reported a detailed methylation study of a 913 bp segment of the genome of HPV-16 encompassing part of the L1 gene and the complete LCR in the cell line CaSki and 115 patient samples. Altogether, we investigated the frequencies of methylation and position of CpGs among a total of 9,090 potential target sites (the sum of the clinical samples and CaSki clones). Our data confirm that HPV-16 genomes are efficiently targeted by the epithelial CpG methylation machinery, raise questions as to the mechanism of this reaction and the biological consequences for the epithelial cell and/or the HPV-16 life cycle.

We did not detect an HPV-16 L1-LCR segment that was completely methylated. A past study, involving one of us (2), observed among 15 clinical samples five that seemed to be completely methylated in this segment, but our present data are more representative of HPV-16 genomes in situ, as the past findings were based on a shorter segment and on direct sequencing, which may have scored mixtures of HPV-16 DNAs that were methylated or unmethylated in the same CpG position as completely methylated.

We measured a lot of "noise" in HPV-16 DNA methylation, i.e. there are no sites that are always or never methylated nor are there conserved stretches of contiguous CpG methylation in the majority of molecules. Methylation does not appear very often at isolated CpGs, but frequently on three to eight or even more flanking CpGs, reminiscent of spread of the modification along a DNA. In spite of these reservations, we observed decreased methylation in three regions, the sites 7270 and 7862, and to a lesser degree the flanking sites 7535 and 7554. It is noteworthy to interpret these positions in the context of known cis-responsive elements and chromatin structures of the HPV-16 LCR. The segment 3' to position 7862, which is often heavily methylated, contains all promoter elements, i.e. the promoter activator Sp1, whose binding is not influenced by DNA methylation (17), the viral factor E2, which is displaced by methylation, and the TATA box. This segment is organized in form of a specifically positioned nucleosome, whose acetylation affects the promoter (38). Position 7862 is located 5' of this nucleosome and coincides with the viral replication origin and a silencer regulated by YY1 and CDP, and the 3' flank of the viral enhancer, activated by AP-1. This region serves as a super-regulator, as YY1 and CDP with their associated HDAC activities and AP-1 with a histone acetylase activity repress and activate HPV transcription in different epithelial layers by influencing the promoter nucleosome and a second nucleosome encompassing the viral enhancer (1,3,26). Methylation and demethylation of the CpG at position 7862 may add another layer of crosstalk to this regulatory region. In addition, position 7862 is part of an E2 binding site that activates HPV replication, and has to remain demethylated to permit another round of replication. DNA methylated at position 7862 would be replication incompetent and eliminated. The fact that position 7862 is close to an Sp1 site conserved among HPVs (43), one is reminded of the ability of Sp1 to suppress methylation from some adjacent CpG elements (7). The properties of this DNA segment may establish a molecular basis for the poorly defined state of "latency" of HPV infections, i.e. presence of HPV DNA in the absence of symptoms, as repression of the enhancer and promoter and accessibility of the replication origin could lead to maintenance replication of the virus without expression of transformation functions.

The enhancer is covered by a second nucleosomes (Fig. 1) and 5' of the enhancer, we found a nucleosomal linker (39), which includes the positions 7535 and 7554. DNAse I sensitivity studies suggested additional specifically positioned nucleosomes 5' of this region, which were separated by another linker around position 7270. These arguments would lead to a proposal that HPV-16 DNA tends to be hypermethylated in nucleosomally organized segments, and undermethylated in nucleosomal linkers, which sounds counterintuitive, but may have functional significance, since nucleosomally incorporated DNA is not protected from but rather still efficiently targeted by CpG maintenance methylation (28). The overlap of specifically positioned nucleosomes with hypermethylated regions is indicated in Fig. 1.

MeCpA and meCpT residues point to de novo methylation in epithelial cells, and our observations confirm that HPV genomes are efficiently targeted in undifferentiated cells in cell culture. While we measured only a total rate of about 0.4% of methylation of these two dinucleotides, the real frequency of de novo methylation is likely much higher, since our approach scored CpGs only in the "maintenance methylation data set" (Fig. 3), but not in the "de novo methylation data set" (Fig. 5), although CpGs are more efficiently methylated de novo than the other two dinucleotides. The occurrence of de novo methylation in a somatic tissue is little studied (44a, 47a) in contrast to embryos and cancer cells (19,32), but not completely surprising, as DNMT3a and b activities have been detected in a variety of normal somatic cells. Strangely, meCpAs, and meCpTs appeared to occur frequently in regions of CpG undermethylation, and we speculate that maintenance methylation is particularly efficient within nucleosomes, and de novo methylation in nucleosomal linkers. While this latter observation is not statistically significant for most sites based on our data set, it is of interest that 14 out of 22 meCpA and meCpT residues observed by Kim and coll. (22) in cell cultures containing HPV-16 occurred in the nucleosomal linkers flanking the positions 7535/7554 and 7862.

Correlations between HPV-16 DNA methylation and pathology. We found methylation of HPV-16 DNA in three different clinical contexts, (i) in asymptomatic

infections (NIL), (ii) in low and high grade CIN, and (iii) in carcinoma, although with differing frequencies.

The methylation of presumably episomal HPV DNA in asymptomatic patients is surprising. We are not aware of a molecular scenario that would be comparable to the persistent maintenance of HPV in asymptomatic infections with the exception of the methylation of a CpG island in the EBNA promoters of Epstein-Barr-Virus. While this alteration occurs in asymptomatic B-cells as well as during carcinogenic progression, it was interpreted to support the tumorigenic process by suppressing of EBNA antigens (33). It is an open question whether our data point to an epithelial defense mechanism against heterologous DNA, or a viral adaption to be maintained in an epithelium without causing a neoplasia. As argued above, we propose that access to the viral replication origin with concomitant suppression of transcription may be an adaption to latency, a molecular state and scientific term, which is not yet formally recognized for HPVs as it is for herpes viruses. Kim and coll. (22) reported an excess of methylated HPV-16 genomes in undifferentiated cells in culture, and a stable relationship between methylated HPV genomes and maintenance of the undifferentiated state may be an embodiment of latency.

The low methylation levels of HPV genomes in CIN lesions, where HPVs replicate normally episomally, may stem from the expansion of the transcriptionally active cell population as expected from the initiation of the neoplastic process together and an increase of the virus genomes (37). This observation can probably be best compared with the transcriptionally highly active productive life cycle of polyoma- and adenoviruses.

The heavy methylation in cancers can be interpreted as confirmation of the "genome defense hypothesis" based on the frequent methylation of chromosomally integrated retroviruses, transgenes (4) and transfected adenovirus genomes and their chromosomal targets (15,41). It is generally known that HPV-16 occurs in carcinomas most often in the chromosomally integrated state (12,36). This does not mean that carcinomas do not contain transcriptionally active HPV genomes, since the example of the cell line CaSki (2) shows that carcinomas often contain HPV genomes in integrated tandem arrays which

are efficient methylation targets, while some of these genomes remain unmethylated and therefore actively expressing the HPV oncogenes (2,45). We caution to hope that methylation inhibitors, resulting in demethylation and therefore activation of repressed tumor suppressor genes, are beneficial for all kinds of carcinomas, as they would actually be tumor promoter in HPV activated lesions (18). While the increased methylation of the L1 gene in our samples may point to a role of methylation in the early-late switch, one may also consider that exonic CpGs have a higher propensity of becoming methylated and may be nuclei from where CpG methylation may spread to promoter regions (25).

Our database leads to hypotheses that we will study experimentally. It is obvious to address the question of whether HPV DNA in capsids is methylated, and if not, by what kinetics it establishes methylation after infection of raft cultures. In preliminary studies of HPV-11, we did not find any methylated HPV-11 genomes in viral particles, but a significant fraction of methylated HPV-11 DNA in newly infected human transplants (3a). In raft cultures with episomal HPVs, one can study the establishment of methylation patterns after integration into the chromosomal DNA. Laser capture microdissection may allow to localize the correlation of methylation patterns with histological sites and pathological features and to analyze by quantitative RNA studies correlations between transcription and methylation patterns. These considerations and our observation that HPV methylation is taxonomically widespread suggest HPV methylation a rich system for basic as well as clinical research approaches.

ACKNOWLEDGEMENTS.

This research was supported by NIH grant ROI CA-91964 and by funds from the Chao Family Comprehensive Cancer Center of the University of California Irvine to H.U.B., by a postdoctoral fellowship of the Cancer Center of the University of California Irvine to M.K., as well as by a Collaborative Research Grant from UC MEXUS-CONACYT to I.E.C.M, H.A.B.S, and H.U.B. We would also like to acknowledge technical support by John Huh and the services provided by Hanne Skomedal, tor Molden, and Irene Kraus of Norchip, Norway in the typing of HPV in cervical cancers.

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LEGENDS TO FIGURES.

Fig. 1. Heterogeneity of CpG methylation in HPV-16 genomes derived from CaSki cells. Each vertical set of rectangles represents one of 19 specific CpG dinucleotides, the

number on the top of the bar the position of this CpG in the genome of HPV-16. Each horizontal set of rectangles represents a 913 bp segment of the HPV-16 genome, covering the 3' end of the L1 gene and the complete long control region. Unmethylated CpGs are indicated by white rectangles, methylated by black ones. The two vertical white separators indicate the borders between amplicons, and discontinuities between supposedly different HPV-16 molecules. The upper part of the figure represents three specifically positioned nucleosomes (39), which may overlap with regions of hypermethylation.

Fig. 2. Heterogeneity of CpG methylation in HPV-16 genomes derived from clinical specimens. The figure visualizes the methylation of 19 CpG dinucleotides in the HPV-16 genomes of five independent clones each derived from one of 115 clinical samples, a data base of the methylation status of 8,885 CpG dinucleotides. A: asymptomatic patients; B: CIN; C: cervical cancer. White horizontal rectangles represent an unmethylated CpG, black rectangles meCpGs. Each quintuplet of horizontal lines represents clones derived from one patient. A horizontal line does not indicate a contiguous 913 bp amplicon, but is derived from three different HPV-16 genomes, as indicated by two white vertical bars. These discontinuities were a technical necessity, since the destruction of bisulfite modified DNA did not allow us to generate the analyzed 913 bp fragment in one contiguous amplicon. White indents in this figure identify clones that could be only partially analyzed due to the low amount of sample DNA. Due to the compression of this figure, Mexican, Norwegian and U.S. samples could not be visually distinguished. The Mexican cohort contained 70% genomic AA variants and only 30% E variants (10), while all Norwegian and U.S. samples were E variants. We could not detect any obvious distinction between the methylation patterns of these cohorts and variants.

Fig. 3. Methylation frequencies of 19 CpG nucleotides in the HPV-16 genomes of five independent clones each derived from one of 115 clinical samples. For the overlap of the genomic positions with the L1 gene, 5'LCR, enhancer and promoter, see Fig. 1.

Fig. 4. Methylation frequencies of 19 CpG nucleotides in the HPV-16 genomes of five independent clones each derived from one of 115 clinical samples separated by pathological properties. Grey bars: Asymptomatic patients (no identifiable lesion, NIL); black bars: low and high grade CIN; white bars: cervical cancer.

Fig. 5. Methylation frequencies of CpA and CpT nucleotides in the HPV-16 genomes of five independent clones each derived from one of 115 clinical samples. The figure represents 107 meCpA and meCpT dinucleotides in the same genomic segment that was studied for CpG methylation. The numbers along the x-axis of this figure indicate the positions of cytosine residues that are part of a CpA or CpT dinucleotide as verifiable by comparison with the complete genomic sequence of HPV-16 (24), while the y-axis represents the frequency of finding a methylated C at this position.

"VARIANTES DEL PAPILOMAVIRUS HUMANO Y DESARROLLO DEL CÁNCER DE CÉRVIX"

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Resumen

Los virus del papiloma humano (VPHs) desempeñan un papel central en la etiología del cáncer cervical. Dado que solamente una pequeña fracción de las neoplasias intraepiteliales cervicales (NICs) infectadas con VPHs de alto riesgo progresa a carcinoma cervical invasivo (CI), deben de existir otros factores implicados en el desarrollo de la neoplasia. Un factor importante que emerge es la variación genómica entre los diferentes tipos virales. Tal variación ha sido utilizada para estudiar la distribución geográfica de estos virus, pero cada día hay más evidencia de que ésta pudiera ser determinante para el desarrollo de la enfermedad neoplásica. Los datos obtenidos indican que diversas variantes del virus tienen características bioquímicas y biológicas alteradas y que representan un factor de riesgo adicional en el desarrollo de las NICs y de los tumores. Esto puede ser relevante no solamente para la biología de la infección con VPH y de su asociación con la neoplasia, sino también para la detección y tipificación del virus en la práctica clínica. Y más allá, en las estrategias para el desarrollo de tratamientos y el diseño de medidas profilácticas (vacunas).

Abstract

Human papillomaviruses (HPVs) play a central role in the etiology of cervical cancer. However, only a small proportion of cervical intraepithelial neoplasias (CIN) infected with high-risk HPVs will progress to invasive carcinoma (IC), there should exist additional factors involved in the development of neoplasia. An important emerging viral factor is naturally occurring genomic variation among different HPV types. Such variation has been used to study the geographical distribution of HPVs, but there is increasing evidence that it may be important in determining the risk of development of neoplastic disease. Collected data indicate that different HPV variants have altered biochemical and biological properties and represent an additional risk factor in the development of CIN and IC of the cervix. This may be relevant not only to the biology of HPV infection and its association with neoplasia, but also for the use of viral typing in clinical practice, and furthermore, in strategies for the development of treatments and the design of prophylactic measures (vaccines).

Palabras clave: VPH, variación genómica, cáncer cervical, subtipos
Keywords: HPV, genomic variation, cervical cancer, subtypes

requiere de otros factores además de la presencia del virus. La mayoría de las infecciones se resuelven espontáneamente en un cierto plazo [24] y solamente una pequeña proporción de NICs 1 y 2 progresará CC. El riesgo de progresión de NICs 3 a CI es de al menos el 12%, como ha sido demostrado por estudios de meta-análisis con seguimiento en un rango de 0.5 a 20 años [25][26].

Varios estudios epidemiológicos han identificado factores de riesgo adicionales involucrados en la progresión de NIC 1 a NIC 3 y CI [22][27][28]. Factores virales tales como la persistencia de la infección, la integración del DNA viral en el cromosoma celular del huésped, la expresión de los genes virales E6 y E7 en células replicándose, y la inactivación del gen E2 (debido a la integración viral o por mutación) están involucradas en el desarrollo y mantenimiento del fenotipo transformado; mientras que factores del huésped tales como el genotipo del HLA y polimorfismos en genes celulares, podrían también ser importantes [6][7][10][12][29-31].

Recientemente se ha sugerido que la variabilidad intratípica de los VPHs esta también implicada en la transformación maligna [32][33]. Algunas variantes tienen propiedades biológicas y bioquímicas diferentes [34], consecuentemente, se ha asumido que podrían también diferir en patogenicidad. La observación de que ciertas variantes son más prevalentes en ciertas regiones geográficas no es sorprendente y podría ser explicada por el efecto "fundador", la co-evolución de los VPHs con las razas humanas o por los patrones de migración humana. Esto también podría reflejar un origen potencial para ciertos tipos de VPHs en ciertas regiones geográficas.

Los datos que han sido generados hasta la fecha acerca de las variantes y su participación en el desarrollo de la neoplasia cervical serán ahora discutidos.

Significancia funcional de la variación genómica intratípica.

La región del genoma del VPH requerida para la transformación ha sido mapeada entre el RLC y los genes virales E6 y E7 (ver figura 1) [35]. Las proteínas E6 y E7 interactúan con una amplia gama de proteínas celulares, aunque las mejor conocidas son p53 y pRb [36]. La transcripción de E6 y de E7 es controlada por el promotor y el potenciador en RLC y por la proteína E2. La RLC contiene varios sitios de unión a E2, además de los sitios de unión para varios factores de transcripción celulares [37]. Considerando lo anterior, se puede asumir que las variaciones nucleotídicas en RLC, E2, E6 y E7 pudieran tener repercusiones funcionales.

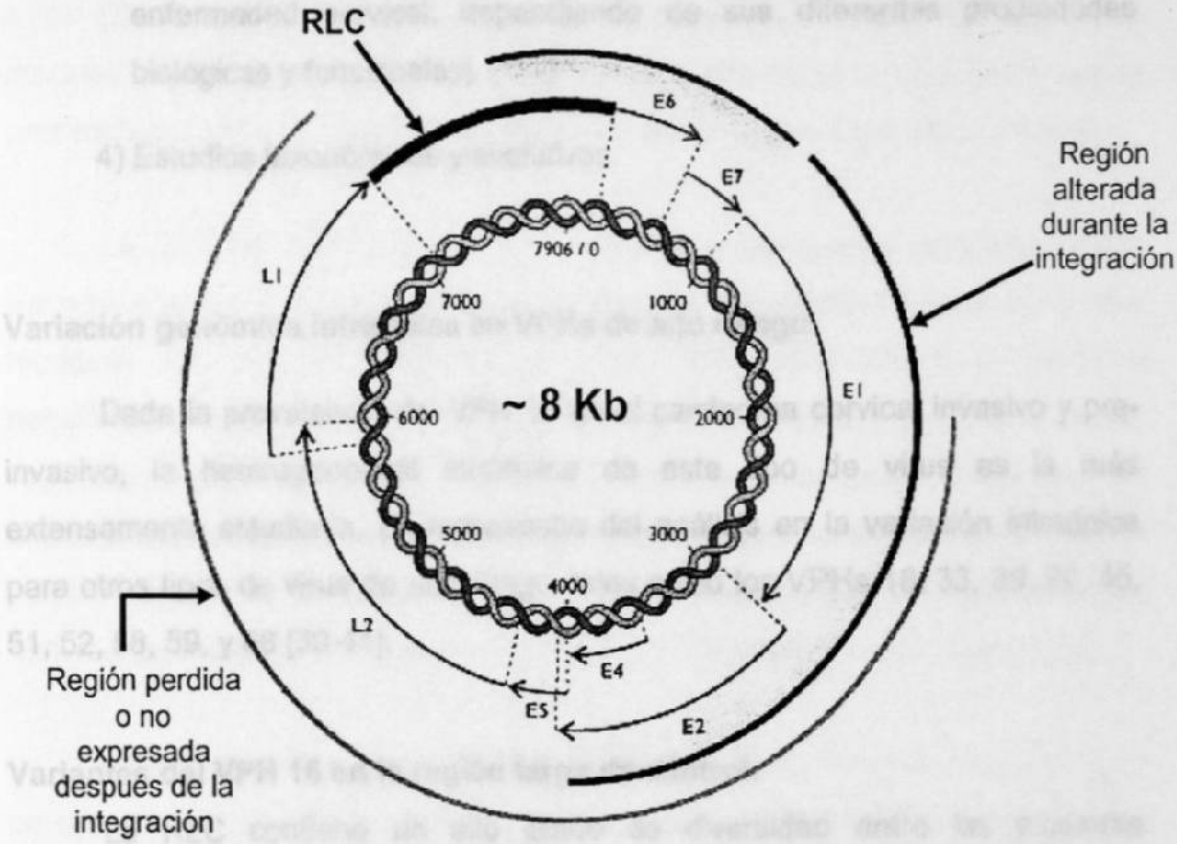

Figura 1. Modelo del genoma del VPH. El genoma de DNA de doble cadena de aproximadamente 8 Kb muestra las tres regiones básicas: a) La región larga de control (RLC), conteniendo secuencias críticas que regulan la transcripción y la replicación de genes tanto virales como celulares, b) Las regiones con marcos abiertos de lectura u ORFs (del inglés, open reading frames) de los genes tempranos (E1, E2, E4, E5, E6 y E7), las cuales codifican para la replicación y transformación celular, y c) Los ORFs de los genes tardíos, los cuales codifican para proteínas estructurales (L1 y L2) [38].

El análisis de la variabilidad genómica de los diferentes tipos de VPHs tiene muchos objetivos. Es importante tener una base de datos para:

1) El desarrollo de herramientas eficientes de diagnóstico y vacunas.

2) Aplicación en los estudios epidemiológicos en los cuales la variación en la secuencia puede ser usada como marcador para monitorear los VPHs en poblaciones definidas.

3) Estudios de relación entre genotipo y fenotipo (por ejemplo ciertas variantes pueden estar positiva o negativamente asociadas con la enfermedad cervical, dependiendo de sus diferentes propiedades biológicas y funcionales).

4) Estudios taxonómicos y evolutivos.

Variación genómica intratípica en VPHs de alto riesgo.

Dada la prevalencia del VPH 16 en el carcinoma cervical invasivo y pre-invasivo, la heterogeneidad intratípica de este tipo de virus es la más extensamente estudiada, en menoscabo del análisis en la variación intratípica para otros tipos de virus de alto riesgo, tales como los VPHs 18, 33, 35, 39, 45, 51, 52, 58, 59, y 68 [39-41].

Variantes del VPH 16 en la región larga de control.

La RLC contiene un alto grado de diversidad entre las muestras analizadas, alcanzado hasta un 5% y contrastando con la mayoría de los genes en otros organismos, para los cuales se observa menor al 2%. Analizando la RLC de una amplia colección de muestras cervicales positivas para VPH 16, Ho y cols. encontraron que la variación en la secuencia formaba cuatro diferentes ramas filogenéticas, a saber:

i) Las variantes asiático-americanas (AA), las cuales son encontradas principalmente en Centro y Sudamérica así como en España.

ii) Las variantes africanas (Af-1 y Af-2), encontradas en África.

iii) Las variantes Asiáticas (As), encontradas en el sureste de Asia.

iv) y Las variantes Europeas (E), encontradas en todas las regiones, excepto África.

Subsecuentes estudios completaron la clasificación de estas variantes a través del análisis de la variación en la secuencia de los genes E6, L1, L2 y de la RLC, agregando la variante Norteamericana-1 [42][43].

Diversos estudios han indicado que la persistencia viral y el desarrollo de lesiones de alto grado y del CI, están no solo directamente asociadas con la presencia del VPH 16, sino que también con las variantes específicas de éste.

La RLC contiene sitios de unión para la proteína viral reguladora de la transcripción E2 [44], así como para factores de trascripción celular, los cuales modulan su función positivamente (Ap1, Sp1, Nf1, Oct-1 y Tef-1) o negativamente (YY1) [45-46]. Los cambios nucleotídicos dentro de esta región reguladora pueden influenciar en la replicación y la transcripción, a través de un efecto en la unión de complejos de proteínas reguladoras con el DNA. Los cambios dentro de los sitios de unión a YY1, juegan un papel especial, pues tienen el potencial de aumentar la expresión de las oncoproteínas virales [47].

Se han aislado algunas variantes europeas de carcinomas cervicales que se caracterizan por tener mutaciones puntuales o deleciones en los sitios de unión de YY1 en la RLC. Comparaciones con la secuencia de referencia europea, revelan que estas variantes tienen aumentada su actividad transcripcional [48]. Este mismo grupo también identificó un sitio de unión a Sp1, el cual se traslapa con el sitio de unión de YY1 en las posiciones 7840-7848. Una mutación puntual dentro de este sitio mostró un incremento de 4.7 veces en la afinidad del promotor P97 por la unión del factor Sp1. Mientras que una

mutante, a la cual le fue eliminada el sitio de unión para Sp1 mostró solamente de 1 a 1.6 veces más aumentada la actividad del promotor. De estos datos se concluyó que la competencia entre Sp1 y YY1 para unirse al DNA juega un papel importante en la represión mediada por YY1 por la unión al sitio 7480-7848. En un estudio realizado en Corea, fueron identificados en muestras de CI que contenian formas episomales de VPH 16, tres mutaciones puntuales en los sitios de unión a YY1 en la RLC. Usando ensayos de transfección, las tres mutantes mostraron de 2 a 4 veces mayor actividad transcripcional que la del tipo silvestre. En contraste, las RLCs de VPHs integrados o episomales sin mutaciones en los sitios de unión a YY1, mostraron niveles similares a la actividad del promotor del prototipo, sugiriendo que las mutaciones en el sitio de unión a YY1 están funcionalmente relacionadas al desarrollo del CC causado por el VPH 16 episomal [49].

En otro estudio, fueron observadas actividades transcripcionales similares en todas las RLCs de todas las variantes E del VPH 16 examinadas [37]. Solamente una muestra AA mostró incrementada la actividad transcripcional casi 1.7 veces más, comparada con la clona de referencia (esta variante mostró cambios en el extremo 3' de la mitad de la RLC entre los nucleótidos 7660 y 7890). De estos datos se ha sugerido que las variantes AA podrían tener un una actividad oncogénica aumentada en comparación con las variantes europeas.

Adicionalmente, aunque, las variantes AA tienen cambios nucleotídicos en dos de los tres elementos de respuesta a la progesterona/glucocorticoides (GREs), estos cambios no resultaron en un incremento en la actividad transcripcional. Aun así, se ha propuesto que la inducción por progesterona podría revelar diferencias funcionales adicionales entre las variantes E y otras del VPH 16 [37].

Variación en la región tardía río arriba del VPH 16.

Como se mencionó anteriormente, la transcripción de genes tempranos ha sido observada en células basales y en capas celulares suprabasales en epitelios normales infectados con VPH, así como también en las células de NICs y en células cancerosas [50-51]. En contraste, los genes virales tardíos L1 y L2 son expresados en la última etapa de la producción viral. El gen L1 codifica la proteína de la cápside mayor y el gen L2 codifica para la proteína de la cápside menor [52-54]. Las proteínas L1 y L2 juntas se ensamblan en una capa de proteína altamente estructurada que rodea al DNA genómico viral de las nuevas partículas virales completas que son secretadas fuera de las células y que contribuirán a la infección. Estas dos proteínas también sirven como antígenos del VPH 16 [55].

La transcripción de los genes tardíos está fuertemente asociada a los queratinocitos diferenciados cercanos a la superficie epitelial [50-53]. Sin embargo, los transcritos de estos genes tardíos también son detectados en capas suprabasales, pero permanecen en el núcleo [51]. La expresión de estos genes tardíos puede ser vista en NIC y CI, pero los niveles detectables de las proteínas L1 y L2 se encuentran más a menudo en las capas celulares que muestran una cierta diferenciación. La producción de las proteínas L1 y L2 en los casos positivos para VPH, se ha observado que se encuentra más elevada (hasta un 87%) en NICs de bajo grado; en forma moderada (29%) en NICs de alto grado, mientras que los niveles mas bajos (19%) son reportados en CI [55]. Por lo tanto, es probable que la expresión de los genes tardíos del VPH 16 en queratinocitos no completamente diferenciados sea regulada por RNA viral, o elementos del DNA y/o por los factores celulares.

Los estudios recientes han proporcionado evidencia directa de la existencia de una región promotora localizada inmediatamente río arriba del ORF del gen L2 del VPH 16 [56-57]: la región tardía río arriba (referida como LUR, por sus siglas en inglés late upstream region). Esta región tiene una función en la regulación de la expresión de genes tardíos dependiente de

diferenciación y se encuentra entre el ORF del gen E5 y el ORF del gen L2, comprendiendo 138 pb. Geisen y Kahn identificaron un elemento de 78 pb como crítico para la actividad del promotor [57]. En un estudio de la variabilidad genómica en el LUR de 50 casos de NICs y CIs positivos para el VPH 16, los resultados demostraron que las variaciones en LUR son más frecuentes que en otras regiones del virus, así como que la frecuencia de la variación del LUR es más alta en el CI que en las muestras con NIC.

Variación intratípica en los genes del VPH 16.

Las proteínas E2, E4, E5, E6, y E7 de los VPHs son importantes por sus propiedades virales tales como: la replicación y la transcripción del DNA, la interacción con el citoesqueleto, la inmortalización y la transformación [58]. La variación en la secuencia de una o más de estas proteínas puede llevar a la alteración de su función biológica y a afectar así el resultado clínico de la infección. La caracterización de la variación en la secuencia nucleotídica dentro de los tipos de VPHs representa un enfoque racional a la identificación de las variantes, las cuales podrían alterar sus funciones biológicas.

Diversos estudios han examinado a los genes E2, E4, E5, E6 y E7 en muestras positivas de HPV 16 y han revelado una fuerte co-variación intergénica en su secuencia [59-61].

Variantes en E6 del HPV 16.

En un estudio en mujeres inglesas con anormalidades citológicas cervicales de menor importancia, se identificó una variante asociada con persistencia viral y progresión a NIC 2 y a NIC 3. Esta variante tenía una base substituida en la región codificante de E6 en el nucleótido 350 (T350G), dando por resultado un cambio aminoácidico de una leucina por una valina (L83V), y se encontraba presente en múltiples linajes virales, incluyendo a las variantes E y a

las AA [62]. Esta variante fue asociada más frecuentemente con CI que con NICs en una población sueca [63]. Sin embargo, en un estudio realizado por los mismos autores, en muestras VPH 16 positivas en mujeres italianas, el prototipo E6 estuvo igualmente distribuido entre NICs y CI, y la variante L83V no segregó con enfermedad de alto grado. En cambio, en esta ultima población, la variante L83V, cuando se encontraba sola, segregaba con NIC, pero no con CI, mientras que las multi-variantes de E6 fueron asociadas a CI [64]. La razón de que esta variante fuese asociada con CI y no con NICs en Suecia, fue debido no a las diferencias funcionales de la proteina E6, si no al hecho de que esta proteina viral en particular evade el reconocimiento inmune del hospedero [65].

En otro estudio con pacientes suecas se demostró que tanto las muestras con NICs, como con cáncer invasor, contenían la misma variante del VPH 16. Sin embargo, no se encontró asociación alguna entre la variante L83V y CI [66]. De la misma forma, en un estudio de los Paises Bajos, no se encontró una asociación con L83V o con ninguna otra variante del E6 y del E7 del VPH 16 en seis lesiones progresando y 34 en un estado no progresivo a enfermedad [67]. Por otra parte, una distribución igual de las variantes del E6 del VPH 16, incluyendo la L83V, fue encontrada en pacientes alemanas con NICs y con CI, sugiriendo que la variación de la secuencia de E6 no es un factor de riesgo para el desarrollo de la enfermedad en esta población [68]. Una distribución igual de la variante de L83V también fue identificada en estudios en el Reino Unido [69-71].

Las discrepancias en los resultados obtenidos en estos estudios pueden reflejar diferencias en el diseño del estudio o en el número de los pacientes examinados. Alternativamente, la oncogenicidad de la variante de L83V podría variar geográficamente, posiblemente debido a las diferencias genéticas entre las poblaciones.

Las diferencias en las actividades funcionales de variantes del gen E6 del VPH 16 ya han sido demostradas [34]. En particular, las diferencias se han identificado en la capacidad de las variantes del E6 de cooperar con la proteína de referencia E7 para la generación de colonias de queratinocitos humanos

resistentes a la diferenciación y a su blanco, la degradación de la proteína p53. Una de las variantes examinadas, la variante 512 (la cual ha sido clasificada como AA), excedió a la proteína de referencia E6 europea en su capacidad para bloquear la diferenciación de los queratinocitos en respuesta al suero y al calcio. En contraste, la variante z84 (clasificada como Af-2) demostró una reducida capacidad de cooperar con E7 en la formación de colonias de resistencia a la diferenciación de los queratinocitos humanos, cuando éstas fueron comparadas con la proteína E6 europea. También se ha observado que la variante Af-2 había reducido su capacidad de dirigir la degradación de p53, comparándola con la variante AA y la variante E de referencia. Por consiguiente, fue propuesto que las actividades biológicas de la proteína E6 del VPH 16, así como su capacidad de inducir la degradación de p53 *in vitro*, están directamente correlacionadas [34]. En otro estudio, fue divulgado que la variante E7 N29S del VPH 16 tenía dos veces más la capacidad de cooperar con la oncoproteína ras en la transformación de los fibroblastos de embrión de rata [72].

Variantes génicas en E7 del VPH 16

Varios grupos investigando diferentes poblaciones han estudiado la variación en la secuencia del gen E7 del VPH 16. Todos estos grupos sugieren que la secuencia prototipo del gen E7 del VPH 16 esta altamente conservada [72][64][68][73].

Sin embargo, en un estudio coreano que investigaba las variantes del E7 del VPH 16 en tejidos normales, NIC 3 y CI, el prototipo fue detectado en solamente el 10% de carcinomas invasivos. La variante más común, N29S (A647G), estaba presente en el 70% de los carcinomas invasivos, indicando que la variación en E7 puede ser un factor de riesgo para la neoplasia cervical en la población coreana estudiada [74].

Variantes del gen E2 del HPV 16

La proteína E2 del HPV juega un papel esencial en la regulación de la transcripción y replicación viral. Esta contiene un dominio N-terminal de activación transcripcional conservado y una región C-terminal responsable para la dimerización y un sitio especifico de unión al DNA [75]. Esta proteína tiene una doble función, pues dependiendo de su configuración, puede activar o reprimir la transcripción del promotor P97 [76]. La integración del DNA viral en el genoma celular lleva a la pérdida de la función de E2, lo cual juega un importante papel en la progresión carcinogénica [77].

La variación en la secuencia en la región E2 del VPH 16 ha sido también analizada [37] y se han encontrado diferencias menores en el potencial de transactivación en las variantes de E2. Además, la transcripción del promotor P97 se vió incrementada en al menos 1.5 veces para cada variante E2 del VPH 16, en comparación con la clona de referencia. Sin embargo, la actividad transcripcional de las variantes en E2 identificadas, difieren levemente.

En un estudio conducido en una población mexicana, las variantes AA del VPH 16 fueron detectadas con frecuencia en CC y estos aislados tenían extensos cambios nucleotídicos en el gen E2 [59]. Mientras que en un estudio reciente con muestras NIC 1 y NIC 3 de una población inglesa, la variante C3684A (T310K) del VPH 16 segregó con muestras NICs 3. En este mismo estudio, ni la variante de T310K, ni cualquier otra variante de E2 del VPH 16 mostró una segregación con la variante L83V del E6 del VPH 16 [71]. En otro estudio se examinó la variación de la secuencia E2 en lesiones NIC 3 y CI, descartándose cualquier correlación entre las variantes de E2, incluyendo T310K, y la presencia de la invasión [78]. Por lo tanto, esta variante puede ser importante en la progresión de NIC 1 para NIC 3, pero factores adicionales parecen ser requeridos para la progresión de NIC 3 al carcinoma cervical invasor.

Variantes del E2 en los HPVs 16 y 18.

En otro estudio, una variante E2 del VPH 18 con potencial oncogénico disminuido fue descrita en lesiones cervicales [79]. En otro estudio, en el cual se analizaron 34 lesiones VPH 16 y 29 positivas para VPH 18 con un amplio espectro histológico de neoplasias cervicales, no se encontró correlación alguna, ya sea entre las variantes del VPH 16 o las variantes del VPH 18, y el grado de lesión. Sin embargo, para VPH 16, las variantes de E2 co-segregaron con frecuencia con la variante L83V del VPH 16, la cual ha sido asociada con persistencia viral. Esta co-segregación fue propuesta como un factor de riesgo adicional para el desarrollo del CC [80].

Variantes en la región RLC del VPH 18

Investigando la variabilidad genética del VPH 18, un estudio detectó 19 mutaciones en la RLC, ocho de las cuales estaban presentes en los sitios de unión a los factores de transcripción [81]. En un estudio posterior, se determinó el significado funcional de dos de las mutaciones encontradas, correspondientes a los nucleótidos 40 y 41 (G40T y G41A) en el sitio Sp1 proximal al promotor de E6/E7 del VPH 18 [82]. La proteína Sp1 se une con alta afinidad al sitio de unión Sp1 mutante más que al del prototipo, y también se observó que la actividad del promotor fue incrementada de 2 a 3 veces más bajo el control de la RLC llevando la mutación G40T y de 4 a 6 veces más llevando la mutante G41A. En general, se ha sugerido que mutaciones puntuales en el sitio de unión a Sp1 dan lugar a una afinidad más alta de la proteína para unirse al DNA y a una actividad más fuerte del promotor de E6/E7.

Variación en la secuencia en los VPH de bajo riesgo

La variación intratípica del VPH en tipos menos prevalentes fue analizada en una colección mundial de especímenes y aportó información acerca de la relación evolutiva inter-tipo [83].

Los VPHs 6 y 11 raramente se encuentran en tumores invasivos. Heinzel y cols. examinaron la diversidad genómica de los VPHs 6 y 11 a partir de muestras de diversas partes del mundo y compararon las funciones del potenciador de las cinco variantes más divergentes. La prueba funcional de un segmento dentro del extremo 5' de la RLC y del potenciador específico del epitelio, indicó diferencias transcripcionales entre los VPHs referidos como 6a, 6b, y dos variantes del VPH 11, así como con la secuencia de referencia del VPH 11 [84-85]. Sin embargo, en otro estudio, la variación en la secuencia en la RLC de muestras positivas para VPH 6, no se encontraron actividades significativamente diferentes entre los promotores responsables de la expresión de las proteínas E6 y E7 [84]. En un estudio posterior, las proteínas E2 de los VPHs 6a y 6b tuvieron actividades transcripcionales idénticas y fueron indistinguibles en su capacidad para estimular la trascripción de un reportero bajo el control de un promotor dependiente de E2, en queratinocitos humanos primarios. De la misma manera, las proteínas E2 de ambas variantes tenían la misma capacidad de reprimir la transcripción *in vivo* [86].

Conclusión

Se han identificado muchas variaciones intratípicas en la secuencia del VPH, con características biológicas y bioquímicas alteradas y se ha sugerido que estas alteraciones afectan la patogenicidad de estas variantes. Sin embargo, los resultados de diversos estudios con respecto a variantes del VPH son contradictorios. La oncogenicidad de las variantes parece estar influenciada geográficamente y por el origen étnico de la población estudiada.

Los estudios aquí revisados proporcionan evidencia que hace pensar que la variación intratípica en la secuencia del VPH puede representar un factor de riesgo importante para el desarrollo de NICs y de CC. Sin embargo se necesitan estudios adicionales sobre las variaciones intratípicas de otros tipos de VPHs y de su papel en la etiología y alteraciones patológicas cervicales, para acabar de sustentar esta interesante hipótesis.

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