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Human Papillomavirus Type 16 Variant Analysis of E6, E7, and L1 Genes and Long Control Region in Biopsy Samples from Cervical Cancer Patients in North India▽

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High-risk human papillomaviruses (HPVs), particularly HPV types 16 and 18 (HPV-16 and HPV-18, respectively), play a cardinal role in the etiology of cervical cancer. The most prevalent type, HPV-16, shows intratypic sequence variants that are known to differ in oncogenic potential and geographic distribution. This study was designed to analyze sequence variations in E6, E7, and L1 genes and the LCR (for long control region) of HPV-16 in cervical cancer patients to identify the most prevalent and novel HPV-16 variants and to correlate them with the severity of the disease. Cervical biopsies from 60 HPV-16-positive cancer cases were analyzed by PCR and DNA sequencing. The most frequently observed variations were T350G (100%) in E6, T789C (87.5%) in E7, A6695C (54.5%) in L1, and G7521A (91.1%) in the LCR. In addition, only one novel variant (T527A) in E6 and four new variants each in L1 (A6667C, A6691G, C6906T, and A6924C) and in the LCR (C13T, A7636C, C7678T, and G7799A) were identified. While E7 was found to be highly conserved, the variant 350G of E6 was the most prevalent in all of the histopathological grades. The majority of LCR variants were found at the YY1 transcription factor binding sites. Interestingly, a complete absence of the Asian lineage and a high prevalence of European lineages in E6, E7, L1, and the LCR (85, 86.7, 67.7, and 63.3%, respectively) indicate a possible epidemiological linkage between Europe and India with regard to the dissemination of HPV-16 infections in India.

Cervical cancer is the major cancer in Indian women and a leading cause of cancer deaths. Every year, more than 130,000 new cases and about 70,000 deaths are recorded. The persistent infection by specific types of high-risk human papillomaviruses (HR-HPVs) is essential for the progression of cervical lesions (6, 11, 31, 60), and women who are infected with HR-HPVs are likely to develop cancer (3, 4, 27, 40). Various studies have demonstrated that more than 70% of invasive cervical cancers harbor HPV type 16 (HPV-16) and HPV-18 (17, 31), and the products of viral transforming E6 and E7 genes have been shown to contribute to tumorigenesis by functionally inactivating two important cellular tumor suppressor proteins, p53 and retinoblastoma (5, 14, 30, 55).

More than 100 types of HPV are known, but only about 30 types are associated with anogenital cancer. According to the Papillomavirus Nomenclature Committee, a new HPV type is defined by a nucleotide sequence variation of more than 10% compared to that of other known HPV types in the E6, E7, and L1 open reading frames. Those differing by 2 to 10% are referred to as subtypes, whereas intratype variants may vary by up to 2% in the coding region and 5% in the noncoding region compared to that of the prototype (2, 10).

On the basis of sequence variations in E6, L1, L2, and the long control region (LCR), HPV-16 variants have been identified and grouped into six distinct phylogenetic branches: E (European), AA (Asian-American), Af1 (African 1), Af2 (African 2), As (Asian), and NA (North American) (54, 56, 57). These variants have been found to show different geographic distributions, with various oncogenic potentials. A number of sequence variations have been reported for HPV-16 E6, E7, and L1 genes as well as in the LCR in cervical cancer (33, 39, 48, 55, 56). Studies also have shown that specific intratype variants may influence the persistence of HPV infection and the progression of precursor lesions to cancer (27, 58, 59). HPV variants also may affect virus assembly, immunologic responses, pathogenicity, p53 degradation, immortalization activity, and the regulation of transcription (15, 18, 24, 25, 37, 51). These variations immediately affect the sensitivity and specificity of different PCR-based genotype diagnostic methods.

Of the two HPV-16 oncogenes E6 and E7, E6 has been found to show more variations than E7, which is relatively conserved (48, 50, 56, 58, 59). The analysis of the L1 gene, which codes for the viral major capsid protein, is of immense importance because of its high diagnostic value. The range of intratype variation observed in this region allows the distinction and assessment of known and novel HPV types (46). This is also an important target for the development of HPV vaccines. Very few reports are available on HPV variants from this region (9, 56). The LCR, which regulates viral transcription, has been found to be the most variable region of the HPV-16 genome (41, 53, 56). Several authors have evaluated the asso-

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ciation of specific HPV-16 variants with viral persistence and the development of cervical intraepithelial neoplasia lesions (3, 27, 34, 50, 52).

In India, as high as 98% of the cervical carcinoma cases are found to harbor HPV infection, and the most prevalent (~80%) type is HPV-16 (7, 8, 22). Although the prevalence of HPV and cervical cancer in India is the highest in the world, there is not much information on HPV variants from different regions of the Indian subcontinent. In the present study, we have examined the sequence variations in E6, E7, and L1 genes and the LCR of the most prevalent HR-HPV type, HPV-16, and correlated them with the age of the women, histopathologic grades of tumors, and oncogenic potential.

MATERIALS AND METHODS

Study population and specimen collection. Out of a total of 90 tumor samples screened by standard procedures (20, 38), 60 HPV-16-positive cervical cancer cases were recruited for the variant analysis in the present study. The tumor samples were from biopsies collected for the routine diagnosis of cervical cancer from the Cancer Clinic of the Department of Gynecology and Obstetrics, Lok Nayak Hospital, New Delhi, India, and Jawaharlal Nehru Medical College, Aligarh, India. Fresh tumor biopsies were collected in chilled phosphate-buffered saline (PBS) and stored at −70°C in a deep freezer for further processing later. Informed consent was obtained from all patients, and the study was approved by the institutional ethical committee. The diagnosis of histopathological grades was done independently by two pathologists. Discrepancies were resolved by a third pathologist to arrive at a final decision.

DNA extraction and typing of HPV. High-molecular-weight genomic DNA from cervical biopsies was isolated by the standard method of proteinase K digestion and phenol-chloroform extraction that is routinely followed in our laboratory (8, 20).

The detection of HPV and the typing of HR-HPV-16 and HR-HPV-18 were carried out using consensus and type-specific primers described earlier (20, 38). The amplification of the β-globin gene served as the internal control. PCR was performed using the in-house PCR protocol that is routinely followed in our laboratory (7, 8, 17). Briefly, the method involved a 25-μl reaction mix containing 100 to 200 ng DNA, 10 mM Tris-Cl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 12.5 μM of each deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP), 5 pmol of each oligonucleotide primer, and 0.5 U *Taq* DNA polymerase (Applied Biosystems). The temperature profile used for amplification was an initial denaturation at 95°C for 5 min, followed by 30 cycles with denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, which was extended for 4 min in the final cycle. The oligonucleotide primers were synthesized in an automated Applied Biosystems DNA synthesizer (model 381A; Foster City, CA) and purified by high-pressure liquid chromatography.

Variant analysis of E6, E7, and L1 genes and the LCR of HPV-16 by PCR and direct sequencing. HPV-16 E6-, E7-, and LCR-specific PCRs were performed with the following specific primers: for HPV-16 E6 (nucleotide [nt] 83 to 559) with an amplicon size of 476 bp, 5′-GAA ACC GGT TAG TAT AAA AGC AGA C-3′ and 5′-AGC TGG GTT TCT CTA CGT GTT CT-3′; for E7 (nt 562 to 858) with an amplicon size of 296 bp, 5′-CCA TAA TAT AAG GGG TCG GTG GA-3′ and 5′-TTT TTC CAC TAA CAG CCT CTA CAT-3′; for the LCR (nt 7437 to 7456 and nt 119 to 147) with an amplicon size of 617 bp, 5′-CCA TTT TGT AGC TTC AAC CCG-3′ and 5′-AAG TGT GGT AAC TTT CTG GGT CGC TCC TG-3′(54). The L1 gene was amplified partially (450 bp) using MY 11/MY 09, the consensus primer, with sequences 5′-GCM CAG GGW CAT AAY AAT GG-3′ and 5′-CGT CCM ARR GGA WAC TGA-3′, where M = A and C, W = A and T, Y = C and T, and R = A and G (38).

For variant analysis, the PCR products were directly sequenced on an automated DNA sequencer (310 ABI Prism genetic analyzer; Applied Biosystems) according to the manufacturer's protocol. PCR products first were purified using an ammonium acetate-ethanol precipitation method to remove unused deoxynucleoside triphosphates and primers and then cycle sequenced using the BigDye Terminator sequencing ready reaction mix (Applied Biosystems) on a Gene Amp PCR 9700 (Applied Biosystems). The raw data were collected using ABI Prism 310 collection software, analyzed using sequencing analysis software (version 3.4.1) on a Macintosh operating system (version 9.1), and compared to the sequence of GenBank/EMBL/DDBJ sequence no. U89348. All of the samples were reverse sequenced to corroborate the findings.

TABLE 1. Association of histopathological diagnosis with variants of E6, E7, the LCR, and L1

Gene	No. (%) of cases exhibiting the prototype or variant gene according to carcinoma type				*P*
	WDSCC (n = 22)		MDSCC[b] (n = 38)		
	Prototype	Variant	Prototype	Variant	
E6	7 (31.8)	15 (68.2)	13 (34.2)	25 (65.8)	0.84
E7	19 (86.4)	3 (13.6)	33 (86.8)	5 (13.2)	0.95
LCR	4 (18.2)	18 (81.8)	11 (28.9)	27 (71.1)	0.35
L1[a]	6 (60.0)	4 (40.0)	14 (66.7)	7 (33.3)	0.71

[a] Variant analysis for L1 was done for only 31 cases (WDSCC, n = 10; MDSCC, n = 19; and PDSCC, n = 2).

[b] Two cases of PDSCC showing only prototype sequences were grouped with MDSCC cases.

Identification of variants. Variants were identified using the prototype sequence (HPV-16R), which belongs to the European lineage, as the standard for comparisons and nucleotide position numbering (32), and variants were classified into the six major branches European (E), Asian-American (AA), Asian (As), African 1 (Af1), African 2 (Af2), and North American (NA), as described by Yamada et al. (56).

Statistical analysis. The statistical analysis was performed using the χ^2 test to determine the association between HPV-16 variants and the age and histopathologic status of the cancer patients. Fisher's exact test was employed for comparisons of small numbers. A P value of less than 0.05 was considered statistically significant.

RESULTS

Out of 90 cervical tumor samples screened with L1 consensus primers, 65 (72.2%) were positive for HPV, and further genotyping with type-specific primers revealed 92.3% (n = 60) positivity for HPV-16 and 7.7% (n = 5) positivity for HPV-18. All 60 patients recruited were exclusively infected with HPV-16, and none of them showed the presence of HPV-18. The patients were 30 to 80 years old, with a mean age of 51.3 years (standard deviation, 12.8). The patients were divided into two age groups: 30 to 50 years and more than 50 years. All of the cases were of squamous cell carcinoma (SCC); 23 (38.3%) were well-differentiated (WDSCC), 35 (58.3%) were moderately differentiated (MDSCC), and 2 (3.4%) were poorly differentiated squamous cell carcinomas (PDSCC). The E6, E7, and L1 genes and the LCR were analyzed for nucleotide variations and were compared to the prototype sequence HPV-16R of the E lineage.

Association between HPV variants and histopathological grades. For the E6 gene, variants were found to be more common than the prototype in both WDSCC (68.2%) and MDSCC (65.8%) cases (the MDSCC cases include two PDSCC cases), while the prototypes of E7 (86.4 and 86.8%, respectively) and L1 (60.0 and 66.7%, respectively) (Table 1) were more common than variants. The frequency of LCR variants also was found to be higher in WDSCC and MDSCC (81.8 and 71.1%, respectively) than that of the prototype (Table 1).

E6 sequence variations. Nucleotide sequences of the complete E6 open reading frame in all 60 cancer patients screened were compared to the HPV-16 reference sequence. Forty-two (70%) patients showed seven different types of E6 gene mutations, of which four, G145T, C335T, T350G, and T527A, were missense mutations: glutamine→histidine (Q14H), histidine→tyrosine (H78Y), leucine→valine (L83V), and serine→

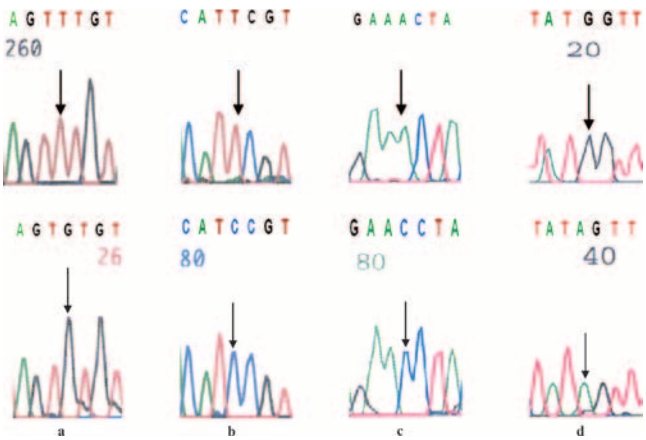

FIG. 1. Sequencing electropherogram showing frequently detected nucleotide variations in different genomic segments of HPV-16. (a) E6 T350G; (b) E7 T789C; (c) L1 A6695C; and (d) LCR G7521A. The upper panel shows prototype sequences, while the lower panel shows variant sequences (indicated by arrows).

threonine (S142T), respectively. The remaining three, T286A, A289G, and A532G, led to silent mutations at codons 61, 62, and 143 for amino acids alanine, valine, and serine, respectively. The most frequently observed mutation was the European variant T350G (Fig. 1a), which was detected in all 42 (100%) patients either alone (34 [81%]) or along with other variants (8 [19%]) (Fig. 2). Additional E6 gene variations were observed only in those cases that showed the 350G variation, and no case with the 350T sequence had any nucleotide change. The majority of cases (85%) were found to be of E lineage, 10% belonged to branch NA, 3.3% to AA, and none to As, Af1, or Af2 (see Table 3). Interestingly, a novel mutation, T527A, was recorded along with 350G (Fig. 2) for the first time.

E7 sequence variations. The E7 gene revealed only five nucleotide variations in 8 (13.3%) patients, whereas the other 52 (86.7%) cases showed the prototype sequence. All of the variants were silent nucleotide alterations; one was a transversion, and the other four were transition substitutions. The silent mutations detected were G666A, T732C, T789C, T795G,

	E6							E7					LCR																	L1												
	145	286	289	335	350	527	532	666	732	789	795	828	13	7521	7636	7669	7678	7689	7714	7729	7743	7764	7786	7792	7799	7826	7834	7839	7886	6667	6691	6695	6721	6803	6854	6865	6889	6906	6924	6964	6970	6994
REF	G	T	A	C	T	T	A	G	T	T	T	T	C	G	A	C	C	C	T	A	T	C	C	C	G	G	G	A	C	A	A	A	G	A	C	C	T	C	A	A	C	G
Scx 1	T	A	G	T	G	-	-	-	-	-	-	-	-	A	-	T	-	A	-	C	-	T	T	-	-	-	-	-	-	-	-	C	A	-	T	T	-	T	-	-	T	A
Scx 2	T	A	G	T	G	-	-	-	-	C	G	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	C	A	-	T	T	-	T	-	-	T	A
Scx 3	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Scx 4	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Scx 5	T	A	G	T	G	-	-	-	-	-	-	-	-	-	-	T	-	A	-	C	G	T	T	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-
Scx 6	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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Scx 8	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Scx 9	T	A	G	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Scx10	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-
Scx11	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Scx12	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Scx13	T	A	G	-	G	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Scx14	T	A	G	T	G	-	G	-	-	C	G	-	-	-	-	T	-	A	-	C	-	T	T	-	-	A	T	C	-	-	-	C	A	-	T	T	-	-	C	-	T	A
Scx15	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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Scx18	-	-	-	-	G	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Scx19	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Scx20	-	-	-	-	-	-	-	-	-	C	G	-	-	A	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	C	A	-	T	T	-	-	-	-	T	A
Scx21	-	-	-	-	G	-	-	-	C	C	G	-	-	A	-	-	-	A	-	C	G	T	-	-	-	-	-	-	G	-	-	C	A	-	T	T	G	-	-	-	T	A
Scx22	-	-	-	-	G	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Scx23	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-
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Scx26	-	-	-	-	-	-	-	-	-	-	-	-	T	A	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-
Scx27	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-
Scx28	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-
Scx29	-	-	-	-	-	-	-	-	-	-	-	-	-	A	C	-	-	-	-	-	-	-	-	-	A	-	-	-	-	C	-	C	-	-	-	-	-	-	-	-	-	-
Scx30	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	A	-	C	G	T	T	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-
Bcx31	-	-	-	-	G	-	-	-	-	-	-	-	-	A	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bcx32	-	-	-	-	G	-	-	-	-	C	-	C	-	A	C	-	-	-	-	-	-	-	-	-	A	-	-	-	-													
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Bcx35	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-													
Bcx36	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-													
Bcx37	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-													
Bcx38	-	-	-	-	G	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-													
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Bcx42	-	-	-	-	G	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-													
Bcx43	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-													
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Bcx47	T	A	G	T	G	-	G	-	C	C	G	-	-	A	-	T	-	A	-	C	G	T	T	-	-	-	-	-	G													
Bcx48	-	-	-	-	G	A	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-													
Bcx49	-	-	-	-	G	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-													
Bcx50	T	A	G	T	G	-	-	-	-	C	-	-	-	A	-	T	-	A	-	C	-	T	T	-	-	A	T	-	-													
Bcx51	-	-	-	-	G	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-													
Bcx52	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-													
Bcx53	-	-	-	-	G	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-													
Bcx54	-	-	-	-	G	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-													
Bcx55	-	-	-	-	G	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-													
Bcx56	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-													
Bcx57	-	-	-	-	G	-	-	-	-	-	-	-	-	A	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-													
Bcx58	-	-	-	-	G	-	-	-	-	-	-	-	-	A	-	-	T	A	-	C	G	T	T	-	-	-	-	-	G													
Bcx59	-	-	-	-	G	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-													
Bcx60	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-													

FIG. 2. Nucleotide sequence variations among the HPV-16 isolates. The nucleotide positions of E6, E7, the LCR, and L1 at which variations were observed are written across the top. The identification codes of the samples are indicated on the left. REF, the HPV-16 reference DNA sequence. For each variant sequence, positions that do not vary from those of the HPV reference sequence are marked with dashes.

TABLE 2. Novel variants of HPV-16 E6, L1, and the LCR according to nucleotide position, variant, altered amino acid, and mutation

Gene	Nucleotide position	Nucleotide		Amino acid		Codon no.	Type of mutation[a]	No. (%) of mutations
		Prototype	Variant	Original	Altered			
E6	527	T	A	Serine	Threonine	142	M, Ts	1 (2.4)
L1	6667	A	C	Serine	Serine	343	S, Tv	2 (18.2)
	6691	A	G	Serine	Serine	351	S, Ts	1 (9.1)
	6906	C	T	Serine	Phenylalanine	423	M, Ts	2 (18.2)
	6924	A	C	Glutamine	Proline	429	M, Tv	1 (9.1)
LCR	13	C	T				Ts	2 (4.4)
	7636	A	C				Tv	2 (4.4)
	7678	C	T				Ts	1 (2)
	7799	G	A				Ts	2 (4.4)

[a] M, missense; S, silent; Ts, transition; Tv, transversion.

and T828C for the amino acids glutamic acid (E), phenylalanine (F), isoleucine (Il), threonine (T), and isoleucine (Il) at codons 35, 57, 76, 78, and 89, respectively. E7 sequencing data indicated that 86.7% belonged to branch E (prototype), 11.6% to Af2, 1.7% to As, and none to NA, AA, or Af1 (see Table 3).

L1 sequence variations. A partial sequence of the L1 gene was screened using a 450-bp amplicon for samples from 31 cervical cancer patients. A total of 13 nucleotide variations were detected in 11 (35.5%) patients, while the majority (20 [64.5%]) showed the prototype sequence. Of 13 variants, 5 (38.5%) led to missense mutations and 8 (61.5%) led to silent mutations.

Of four variants found independently (Fig. 2), two were silent mutations, A6667C and A6691G, coding for serine at codons 343 and 351, whereas A6803T and A6964C led to the missense mutations threonine→serine (T389S) and lysine→asparagine (K442N). The most frequently detected L1 variation, A6695C (Fig. 1c), was found in six (54.5%) cases. Interestingly, four novel L1 variants consisting of two silent mutations, at A6667C and A6691G, and two missense mutations, serine→phenylalanine (S423F) at C6906T and glutamine→proline (Q429P) at A6924C, were observed (Table 2). The novel L1 variants that exhibited silent mutations had only prototype sequences at E6 and E7 gene regions and the LCR (Fig. 2). The L1 sequence data showed that 67.7% belonged to branch E, 3.2% to branch E (G131), 12.9% to AA, 6.5% to NA, and none to As, Af1, or Af2; 9.7% were new variants (Table 3).

TABLE 3. Frequency distribution of HPV-16 variants in different phylogenetic lineages

Lineage	No. (%) of variants for gene:			
	E6 (n = 60)	E7 (n = 60)	L1 (n = 31)	LCR (n = 60)
E (P)		52 (86.7)	21 (67.7)	38 (63.3)
E - P (350T)[a]	18 (30)			
E - P (350G)[b]	33 (55)			
E (G131)			1 (3.2)	
AA	2 (3.3)		4 (12.9)	8 (13.3)
NA	6 (10)		2 (6.5)	
Af1				7 (11.6)
Af2		7 (11.6)		2 (3.3)
As		1 (1.7)		
New variant(s)	1 (1.7)		3 (9.7)	5 (8.3)

[a] Prototype of E6.
[b] Typical HPV-16 variant of E6.

LCR sequence variations. The LCR of HPV-16 showed the highest number of nucleotide variations in various known and unknown regulatory binding sites of a number of cellular and/or viral transcription/regulatory factors. A total of 45 (75%) variant cases that were analyzed showed 17 different point mutations (nine transitions and eight transversions). Of these, seven (G7521A, A7636C, C7689A, T7714G, C7792T, G7826A, and A7839C) were detected in the known binding sites for various transcription factors, such as YY1, AP1, TEF1, NF1, and Oct-1. The most commonly observed LCR variation was the transition substitution G7521A (Fig. 1d), which was detected in 41 (91.1%) patients at one of the many YY1 binding sites. This mutation occurred alone in as many as 22 (53.7%) cases (Fig. 2).

Another frequently observed LCR variation was a transversion mutation, T7714G, detected in nine (20%) cases, mostly along with G7521A, which is located in the NF1 transcription factor binding site. Two other LCR variants, T7743G and C7764T, were detected upstream of the NF1 binding site in 11.1 and 22.2% of cases, respectively. We report here four novel LCR variants, C13T, A7636C, C7678T, and G7799A (Table 2), of which three were transition mutations and one (A7636C) was a transversion mutation located at the AP1 binding site.

A phylogenetic classification of LCR variants was found to exhibit the same pattern as that observed for E6, E7, and L1, showing that a majority (63.3%) belonged to branch E (P), 13.3% to AA, 11.6% to Af1, 3.3% to Af2, and none to NA or As. Four (8.3%) novel LCR variants were observed for the first time from India (Table 3).

DISCUSSION

HPV variant data are important in developing HPV diagnostics, vaccines, and other therapeutic approaches to control virus-induced diseases. HPV-16 variants have been shown to have different biological as well as biochemical effects, resulting in altered oncogenic potentials (1, 47, 59). The oncogenicity of distinct HPV variants also may differ between geographical regions because of differences in the population related to the distribution of HLA alleles (28).

Nucleotide sequence variations observed in E6, E7, and L1 genes and the LCR of HPV-16 in 60 cervical carcinoma cases showed a high prevalence of 350G E6 variants (the prototype is

350T) in all of the histopathologic grades (WDSCC, MDSCC, and PDSCC). This is in good agreement with previous reports (1, 59), which demonstrated an increased frequency of 350G E6 variants in cervical cancer leading to an amino acid change of leucine to valine at position 83 (L83V), and this has been associated with the progression of cervical lesions (59). In contrast, several authors failed to find a significant role of the E6 variants in cervical cancer (19, 33, 50). A study of the prevalence of the E6 gene mutations in a subset of south Indian cervical cancer patients found only 20% of cancer patients with 350G variants, which is in sharp contrast to the present study, which shows that 78% of the patients had 350G variants. Our results, however, are in good agreement with those of a study by Sathish et al. (39) from the southeastern part of India.

We have observed that nucleotide positions 83 to 144, 146 to 285, and 351 to 526 are the most conserved regions of HPV-16 E6, which seems to be important for silencing E6 expression using short interfering RNA and/or ribozyme treatment. Also, the amino acids located in these regions may play an important immunogenic role in new vaccine strategies (21). The amino acid substitutions analyzed in this study are Q14H, H78Y, and L83V, and they also were observed by Stöppler and his group (47). Most interestingly, 100% distribution (42/42) of the HPV-16 E6 E variant 350G was detected; it was found either alone (81%; 34/42) or in combination with additional E6 gene mutations. The E6 variations G145T and C335T that were detected with 350G also have been observed in AA, NA, Af1, and Af2 lineages. A novel E6 gene mutation, T527A (S142T), that replaced serine with threonine was observed in only one (2.4%) case. In sharp contrast to a report from China that showed a high prevalence of the HPV-16 As lineage (55), our results as well as the reports of other authors from India (36, 39) suggest the complete absence of the As lineage in India.

HPV-16 E7 gene mutation has been reported to be rare in various geographic and ethnic populations around the world (16, 36). However, several studies, particularly from Japan, Korea, and Indonesia, have reported a high frequency of E7 gene mutation (65 to 75%) in cervical cancer patients (9, 45). Interestingly, a recent report from China showed that 100% of cervical cancer cases had nucleotide variations in the HPV-16 E7 region (55). In contrast, we observed a highly conserved E7 region; as high as 86.7% of patients showed no sequence variation, and only 13.3% showed variation in the form of silent mutations, thus having no effect on proteins. Because of the conserved E7, it can be far more easily targeted in order to silence its effects at the RNA or protein level. The most frequently reported E7 variation, A647G, also has been found to be completely absent in India. Any change in the two transforming genes E6 and E7 may lead to altered biological function and oncogenicity of the proteins encoded, which can affect the natural history of HPV infection (13).

Examinations of the L1 gene also did not reveal any significant variations, and 64.5% (20/31) of the samples showed the prototype sequence. Very few reports are available on the HPV-16 L1 variants (9, 53, 54, 56), and none are from south Asia. Interestingly, the common L1 variant T6862C observed in all previous studies was not detected; instead, four novel nucleotide variations, two silent and two missense mutations, at codons 343, 351, 423, and 429 were detected (Table 2). This is indicative of the hypervariability of the L1 gene in this region. The importance of the most frequently observed L1 gene variation, A6695 (T353P), lies in the fact that a polar uncharged amino acid (threonine) is replaced by a nonpolar aliphatic amino acid that may have an effect on the structure or function of the L1 protein, which may play an important role in immune recognition and vaccine development strategies. Amino acid changes among molecular variants also have been shown to affect the efficiency of HPV-16 L1 proteins to self assemble into virus-like particles (25, 49). This variability could lead to conformational changes within epitopes relevant for viral neutralization (44).

The LCR, which contains the upstream regulatory region, is the binding site of various cellular and viral transcription factors that either activate or suppress the p97 promoter activity, which regulates the transcription of various HPV-16 genes, especially the E6 and E7 oncogenes. It has been reported to be the most variable segment of the HPV-16 genome in different populations (23, 26, 53, 56). We observed a similar pattern, as 75% (45/60) of patients had 17 different LCR variants, of which four (C13T, A7636C, C7678T, and G7799A) were found to be novel types, including an A7636C substitution that was detected in the AP1 binding site (4.4%). Since LCR is the major site for the transcriptional control of the virus, studies are under way to correlate the observed alterations with the biological/biochemical properties and different clinicopathological features of the disease. The number of nucleotide alterations was highest for the LCR, followed by the L1, E6, and E7 genes, and this is in good agreement with results from a previous report (56). The most frequently observed mutation in the LCR was G7521A, which is located at the YY1 binding site, was found in as many as 91% of cases, and causes the repression of HPV transcription. This mutation has been found to be uniformly distributed in the majority of cervical cancer patients throughout the world (26, 41, 53, 56) but is not found in asymptomatic carriers (41). Mutations in the YY1 binding sites have been shown to promote the p97 promoter activity by three- to sixfold (12, 29). It also has been shown to quench AP1 activity, thereby repressing HPV-16 transcription (35). The role of mutation at the YY1 binding site in cancer cases assumes a greater importance, as this mutation is absent from noncancerous lesions and asymptomatic carriers (42). The presence of mutations in the binding sites of various transcription factors or in their close vicinities is intriguing, since these factors enhance the transcription activity of HPV-16 oncogenes. A low frequency (4.4%) of mutation found in the Oct-1 binding site appears to be important, since Oct-1 is an important part of the basal transcriptional machinery and is known to down-regulate HPV expression (43).

Most interestingly, when the distribution of HPV-16 variants with respect to E6, E7, and L1 genes and the LCR was compared to that of different lineages, an extremely high prevalence of the E lineage (85, 86.7, 67.7, and 63.3%, respectively) and the complete absence of the As lineage (36, 39) were observed. The occurrence of AA and NA classes in this study is in accordance with other studies from the southern part of India (56). It is important that HPV variants are correlated with the severity of lesions through follow-up data to elucidate the biological significance of HPV-16 variants during cervical carcinogenesis, thus delineating the utility of such correlations

in developing reliable diagnostics and effective therapeutics, including vaccines against HPV.

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