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JC9813—A Putative Novel Human Papillomavirus Identified by PCR-DS

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Papillomaviruses consist of more than 130 viral types described so far. Most of them are human papillomaviruses (HPV) of supergroup A, demonstrating ano-genital tropism and characterized as etiological agents for benign and malignant cervical lesions in women. A PCR-direct sequencing (PCR-DS) approach with P-33 labeled dideoxynucleotides was used to detect and type human papillomaviruses in cervical biopsies. One novel sequence was identified in a LSIL (low-grade squamous intraepithelial lesions) specimen from an HIV-positive English Canadian patient. The structure of the viral gene L1 was determined, yielding a putative novel HPV type of supergroup A (clade A8) named JC9813.

Key Words: **new HPV type; papillomavirus; L1 gene sequence; clade A8, PCR-DS.**

Papillomaviruses play a well-known role in cancer (1). Human papillomaviruses (HPV) of supergroup A exhibit ano-genital tropism and are referred to as causative agents in cervical intraepithelial neoplasia (CIN) and squamous cell carcinoma. Cervical cancer screening and patient management increasingly involve HPV testing. The latter is based on detection of viral DNA by Southern blots, dot blots, in situ hybridization, PCR, and solution hybridization (2). These methods cannot characterize novel HPV sequences, for which DNA sequencing is required. Until now, the majority of novel HPV sequences were obtained after PCR, cloning and sequencing. Cloning is a time-consuming step, which could potentially be eliminated by PCR-direct sequencing (PCR-DS). However, direct sequencing of PCR fragments until recently yielded lower quality of the sequencing reactions, characterized by frequent "strong stops". The secondary structure-related sequencing ambiguities could be partially avoided with cycle sequencing. Unfortunately when cycle sequencing is performed with labeled primers or labeled deoxynucleotides, it still requires reamplification and cloning to resolve some difficult to interpret sequence results (3).

We addressed the molecular diagnosis of HPV infection by PCR-DS with [α-^{33}P]-labeled dideoxynucleotides. One hundred and five specimens were tested by both in situ hybridization and PCR-DS methods and the latter proved superior in sensitivity and specificity (manuscript submitted). We present here the final results of our work on JC9813, a putative novel HPV sequence identified by the PCR-DS in the above-mentioned comparative study.

MATERIALS AND METHODS

Specimens. Cervical biopsies (see below) were taken in 1997 from a 38-year-old female patient of English Canadian origin, HIV-positive since 1988, with concurrent health complications, both HIV-related (arthritis) and non-HIV-related (prolapsus uteri), the latter warranted a hysterectomy in 1997, at which time the biopsy was taken. Condylomatous changes of the cervix were first diagnosed in 1986. Informed consent about the use of the pathological specimens for clinical diagnostic and research purposes was obtained according to the procedures established at Ste-Justine Hospital, University of Montreal, and by the Quebec's Minister of Health.

Histological examination. Three exocervical and one endocervical biopsies were examined histologically, after routine formalin fixation, paraffin embedding, and staining with HPS (Haematoxylin-Phloxine-Saffron).

DNA template preparation. Two 5 μm-thin paraffin-embedded cryosections were placed in a microfuge tube and 200 μl solution containing 300 μg/ml Proteinase K, 0.5 % Tween-20, 50 mM Tris-

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HCl, pH 8.5 and 1 mM EDTA was added. The mixture was overlaid with 2 drops of light mineral oil and incubated at 66°C for 3 hours (or overnight). The enzyme was then denatured by incubation at 95°C for 10 min and the solution was centrifuged in a microcentrifuge at 6,000 rpm for 5 min. The aqueous phase was used directly, without purification, as a template for PCR amplification.

HPV PCR. After verification for the presence of DNA by PCR amplification of a 125 bp human microsatellite DNA, general consensus primers GP5 (5′-TTTGTTACTGTGGTAGATAC YAC-3′, where Y;LT or C) and GP6 (5′-GAAAAATAAACTGTAAATCATATTC-3′) were used to amplify the corresponding part of the HPV L1 gene. The PCR was performed according to the original reports (4), with the following parameter modifications: initial denaturing at 94°C for 4 minutes; 40 cycles each at 94°C for one minute, 50°C for one minute and 72°C for 30 seconds; final extension at 72°C for 4 minutes. All PCR amplified products were verified by electrophoresis in ethidium bromide-stained polyacrylamide or agarose gel and subsequently used as template for the cycle sequencing. When the sequencing results yielded a putative novel HPV sequence, which differed by more than 10% from the closest known HPV, we designed additional primers to complete the L1 gene sequencing. One additional degenerated primer, MY84 (5′-ACGTBTBYCATATTYTYTTRCA-3′), was designed to cover the 5′-end of the L1 gene, so that the codon for the first methionine would lie downstream of this primer. The International Union of Biochemistry (IUB) codes were used for the mixed bases (R = G or A, K = G or T, S = G or C, W = A or T, M = A or C, Y = T or C, D = G or A or T, V = G or A or C, B = G or T or C, H = A or T or C, N = G or A or T or C). The "reverse" primer MY89 (5′-NNNNYBDDSWACCGAAATCGGT-3′) was synthesized to anneal downstream of the L1 stop codon region in order to amplify the 3′ region of the gene. The sequence information obtained with the above "universal" primers was then used to design new, JC9813-specific primers, based on the sequences of the identified putative novel clone. The names of the primers with the sequence of the corresponding part of the JC9813 sequence are: F108 (5′-ATGCTGGCAGTTCCCGTTTA-3′), R128 (5′-AAAAGGGTGGCCCACAGCAA-3′), F285 (5′-CAGACACCCAGCGTTTAGTA-3′), F347 (5′-GGTGTTGGAATAAGTGGCCA-3′), F1010 (5′-CAACTCGCAGCACTAACTTA-3′), F1 (5′-GTGCATCCACTGAGTCTGTGCTA-3′), R1 (5′-GTCATATGTAGTAGGTAGCACAGA-3′), R1210 (5′-GTCTTCTAATAATGATGCAT-3′), F1240 (5′-TATCCCCTCCAAGCCACGGAT-3′). The anchor sites for all primers are shown in Fig. 1. PCR was performed using standard protocols (5), optimal annealing temperatures were determined experimentally for each pair of primers. In order to avoid primer interference in the subsequent sequencing reaction, lower primer concentration was used in the PCR, usually 5 picomoles per 25 μl reaction.

Sequencing, sequence alignments, phylogenetic trees. The sequencing of purified and non-purified PCR products was performed with [α-^{33}P]2′,3′-dideoxyribonucleoside 5′-triphosphates. For the latter we used the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham Canada, Baie d'Urfé, Québec) and followed the manufacturer's instructions. The template for the cycle sequencing was 1 μl of (non-purified) HPV-specific PCR product, amplified as described above.

Sequence alignments were obtained from four sources: returned results from GenBank's on-line BLAST server (http://www.ncbi.nlm.nih.gov/), MACAW program for Windows (Version 2.0.5, Greg Schuler, NCBI, NIH, Bethesda, MD), MacMolly Tetra software (Soft Gene Gmbh, Berlin, Germany), and downloaded HPV sequence alignments from the on-line HPV database (6).

The phylogenetic tree for most of the published HPV and animal papillomavirus sequences is available from the 1997 compendium on HPV (http://hpv-web.lanl.gov/)(6). We based our analysis on the sequence alignments available from the above on-line HPV database, with the addition of the putative novel HPV type that we have identified. Different phylogenetic methods were employed, as described in the Results section, which are part of software packages PHYLIP (Phylogeny Inference Package, Joseph Felsenstein, Version 3.57c from 1995)(7), MacMolly Tetra (Soft Gene Gmbh, Berlin, Germany), and MacClade.3.04 (Sinauer Associates, Sunderland, MA).

RESULTS

Histological examination. The exocervical biopsies showed mild dysplasia, warranting the diagnosis of LSIL (low-grade squamous intraepithelial lesions).

Detection of HPV genomic sequences by PCR. PCR-DS typing of a putative novel HPV. The PCR reactions were carried with consensus primers GP5/GP6 following the published protocol (4; 8). They were followed by direct sequencing, which identified a putative novel HPV type (sequence diversion from any known HPV type greater than 10%), termed JC9813. In order to further characterize the putative novel type, we expanded our PCR-DS effort with additional degenerated primers as well as with newly designed JC9813-specific primers.

The complete nucleotide sequence of the gene encoding the L1 protein of JC9813 was deposited in GenBank under the accession number AF070938. Attention was paid to overlap of the PCR fragments used as templates, as well as excluding the end-primers from the readout in order to avoid submitting primer sequences with the viral gene deposited in GenBank. Therefore, the 5′- and 3′-most primers (MY84 and MY89) were selected to lie outside of the L1 coding region. The nucleotide and deduced amino acid sequences are represented in Fig. 1. The PCR fragments used as templates, as well as all primer positions are indicated.

The homology between JC9813 and the closest published HPV was studied by sequence similarity searches and alignments. At nucleotide and protein level, the closest sequences are those of HPV43 (GenBank acc. No U12504), HPV7 (GenBank acc. No X74463) and HPV40 (GenBank acc. No X74478). These three viruses are currently grouped in a separate viral clade, known as A8 (6). Some sequence comparison data are shown in Table 1.

JC9813 is only 78 % identical to the closest known HPV type (HPV43) at nucleotide level. The criteria of the Annual Papillomavirus Conference in Quebec City (1995) state that differences between a novel sequence and the established prototypes, which exceed 10 % at the level of the open reading frame for the L1 protein, define a new type (1). Hence JC9813 is a putative novel HPV type.

Phylogenetic analysis. We used the on-line HPV database (6) to download alignments of sequences, to which the novel sequence was added and then processed by various software packages. The resulting tree is shown on Fig.2.

The new HPV type, JC9813, lies within the pre-

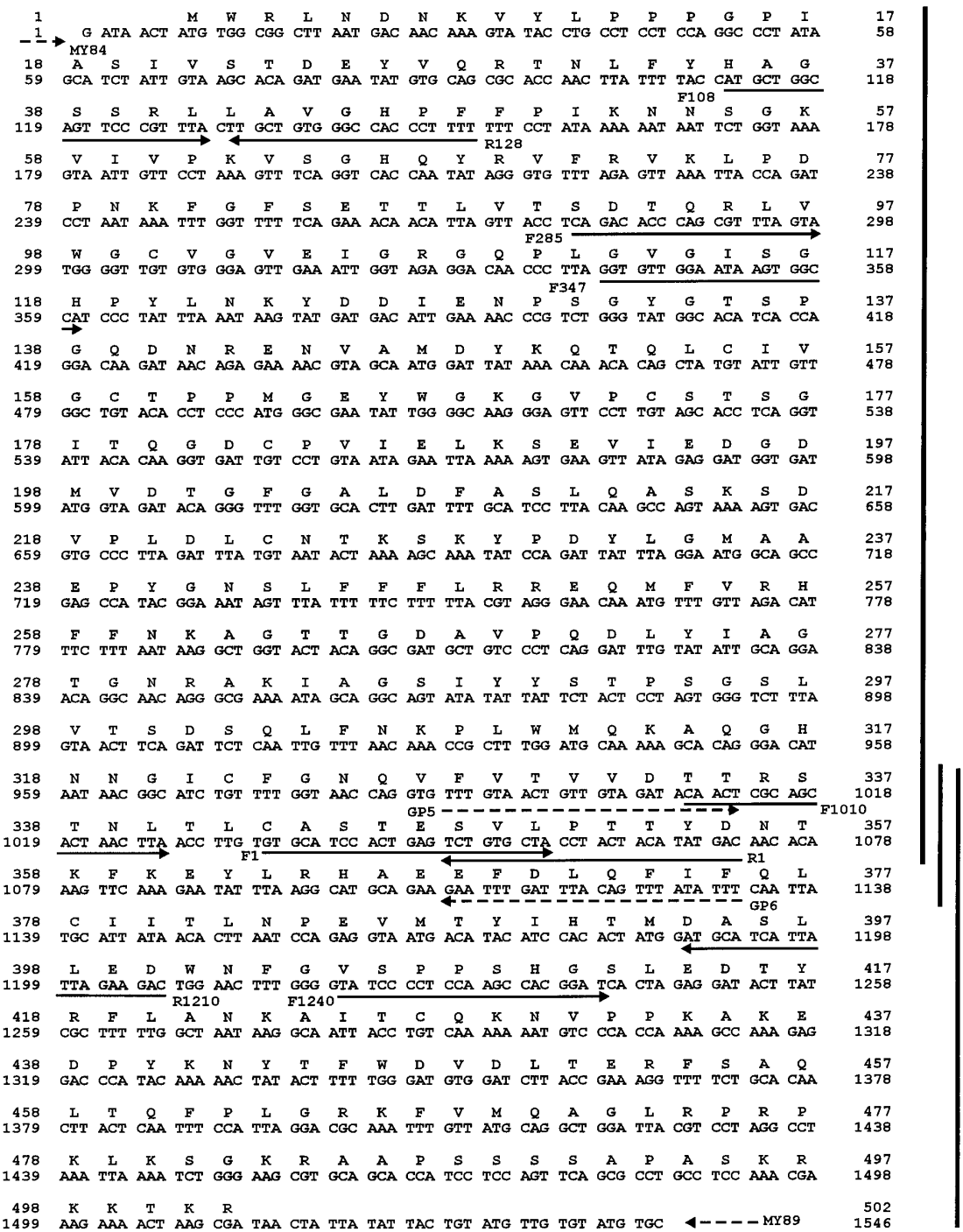

FIG. 1. Nucleotide and deduced amino acid sequences of the L1 gene of the putative novel papillomavirus JC9813. Arrows indicate the orientation of the primer (forward or reverse). Straight horizontal lines signify the JC9813-specific (non-degenerated) primers, while dashed lines are used for the "universal" HPV primers. The vertical lines at the right indicate the PCR products used as sequencing templates.

viously defined clade A8, as shown on the most recent phylogenetic tree, available at http://hpv-web.lanl.gov/, computed on the basis of the neighbor-joining method (NEIGHBOR program of the PHYLIP package, based on Saitou and Nei's neighbor-joining method for distance matrix data). The tree from Fig. 2 was computed using the Fitch-Margoliash and least-squares methods (FITCH program of the PHYLIP package). Essentially the same results were also obtained by parsimony analysis (DNAPARS from PHYLIP, as well as MacClade,

TABLE 1

Identity between JC9813 and Some Close HPV Types in Percentage Points

Identity	JC9813	HPV43	HPV7	HPV40	HPV16	HPV31
Nucleic acid	100	78	73	71	66	66
Protein (total L1)	100	N/A	78	76	66	66
Protein (MY09-11)	100	85	79	78	72	67

Note. Data for HPV43 are available only for the MY09–MY11 consensus region; for all other pairs the full L1 sequence was compared.

data not shown). Results were similar despite the substantial differences in the treatment of sequence "gaps" by these programs.

DISCUSSION

We employed PCR-DS for HPV diagnosis and typing. HPV-specific PCR was followed by direct sequencing of the PCR products with ^{33}P-labeled dideoxynucleotides. This strategy allows us to identify putative novel HPV types. Since a major drawback of the PCR based methods is the difficulty of detecting co-infections with two or more HPV types in the same patient, we were very careful to extensively overlap the amplified PCR fragments, in order to avoid cross-contamination of JC9813 with another type. In fact, in the studied specimen we detected a different HPV type (MM8) when the degenerated primers MY09/MY11 were used as described previously by others (9). We did not detect MM8 with the GP5/GP6 set of primers or with any of the JC9813-specific primers; in addition, the two sequences (MM8 and JC9813) differ all along their L1 genes and proteins and belong to different HPV clades. Some technical improvements were introduced to speed up and simplify the previously known methods. DNA template preparation from cryosections was simplified, we avoided phenol-chloroform extraction and ethanol purification. The use of labeled dideoxynucleotides eliminated the "strong stops" (bands in all four lanes), a serious problem with the conventional (primer- or deoxynucleotide-labeling) methods. Due to the high quality of PCR-DS, in 350 clinical specimens tested so far, a cloning step was not required to resolve ambiguities. Our procedure was further simplified by the use of a small aliquot from the PCR reaction as a template for the sequencing step directly, without purification. The above modifications significantly reduced the time and the cost of our PCR-DS based HPV testing. The method of PCR-DS is more informative than hybridization or RFLP-based methods; it enables unequivocal typing, including instant identification of putative novel types.

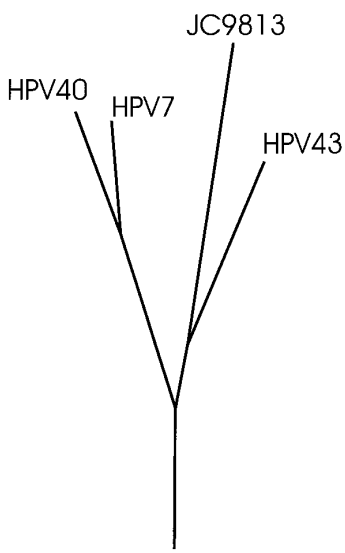

FIG. 2. Phylogenetic analysis of JC9813, HPV43, HPV7 and HPV40. Expanding the clade A8. The most recent update of the phylogenetic tree of all HPV viruses known by the end of 1997 could be consulted at http://hpv-web.lanl.gov/ *(*see also Information for reviewers).* The tree on this figure was drawn with the PHYLIP 3.57c package and is based on the Fitch-Margoliash and least-squares methods.

We detected a putative novel HPV type, JC9813, in a sample from an HIV-positive female patient. HPV infections were reported previously in HIV seropositive patients and large epidemiological studies have found increased cumulative prevalence and persistent HPV infections in seropositive women (10; 11).

The clinical importance of the HPV detection and typing relates to the high or low oncogenic potential of the HPV type(s) found in the clinical specimen. It seems probable that JC9813 is of low oncogenic potential, because two of the three other members of the same clade (HPV40 and HPV43) were reported as low-risk types (8) and type 7 was reported as a causative agent for benign "butcher's" warts (1). In addition, low oncogenic potential for JC9813 would be in accordance with the long history (12 years) of benign cervical lesions in this HIV-positive patient. Based on this single patient and the L1 data, however, a definitive conclusion about the oncogenicity of JC9813 is not possible at present time. In our case the interpretation of the HPV typing did not affect the clinical decision-making about the patient, since she was hysterectomized at the

time of the biopsy due to prolapsus uteri. This precluded also the use of follow-up information for this patient as a method to study the role of JC9813 in human cervical pathology.

PCR-DS does not necessarily require extensive sequence reading for reliable HPV typing. As shown by us elsewhere (manuscript submitted) *(*see also Information for reviewers only)*, a short region of as little as 34 bases adjacent and downstream of the GP5 primer is enough to type all 125 papillomavirus types for which sequences of that region are known to us, including the putative novel type JC9813.

We have previously identified another putative novel HPV type, JC9710 (12). Although both JC9710 and JC9813 originated from HIV-positive women from Quebec (French Canadian and English Canadian, respectively), the two putative novel HPV types are not closely related and they belong to different HPV clades. The finding of two putative novel viral types among the first 80 HPV-positive samples studied by PCR-DS suggests that identification of novel HPV types may still be expected at a relatively high rate.

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