REPORTS

Genomic Variation of Human Papillomavirus Type 16 and Risk for High Grade Cervical Intraepithelial Neoplasia

Long Fu Xi, Laura A. Koutsky, Denise A. Galloway, Jane Kuypers, James P. Hughes, Cosette M. Wheeler, King K. Holmes, Nancy B. Kiviat*

Background: Epidemiologic studies have demonstrated strong and consistent associations between the detection of human papillomavirus (HPV) type 16 DNA and the risk of cervical intraepithelial neoplasia (CIN) and cervical cancer. However, HPV16 is also the most common type of HPV in the normal population, and only a minority of women with HPV16 infection develop cervical cancer. Studies of genomic heterogeneity in HPV16 have demonstrated the presence of multiple variants in all human populations examined to date. It is conceivable that the natural variants of HPV16 in a given population may not have the same biologic behavior. Purpose: This study was designed to determine the association between natural variants of HPV16 and the risk of biopsy-confirmed CIN 2 or 3, the most important precancerous lesions of the uterine cervix. Methods: Prospective studies were conducted among 1) women attending a university and 2) women presenting to the sexually transmitted disease clinic. Subjects were eligible for inclusion in this investigation if the initial cytologic findings did not reveal CIN 2-3 and HPV16 DNA was detected by means of a polymerase chain reaction (PCR)-based method in one or more cervical or vulvovaginal samples. Eligible subjects were followed every 4 months with cervical Pap smears and colposcopic examinations. Women were referred for biopsy if cytology or colposcopy suggested CIN 2-3. Two groups of HPV16 variants, prototype-like and nonprototype-like, were determined by means of single-strand conformation polymorphism (SSCP) analysis of PCR products from the noncoding region of the viral genome. Representative SSCP patterns from HPV16 variants were further characterized by direct DNA sequencing of the PCR products. Relative risks (RRs) and 95% confidence intervals (CIs) were calculated by Cox regression analysis. Results: Prototype-like variants accounted for 79% of the HPV16 detected in university students and 86% of the virus detected in patients presenting to the sexually transmitted disease clinic. CIN 2-3 was confirmed by biopsy in nine of 57 HPV16-positive women attending the university and in 10 of 66 HPV16-positive women presenting to the sexually transmitted disease clinic. Among university students, those with HPV16 nonprototype-like variants were 6.5 (95% CI = 1.6-27.2) times more likely to develop CIN 2-3 than those with prototype-like variants. A similar association was observed among women presenting to the sexually transmitted disease clinic (RR = 4.5; 95% CI = 0.9-23.8). Conclusions: This study suggests that the risk of developing CIN 2-3 is not the same with all variants of HPV16 and that nonprototype-like variants confer a greater risk compared with prototype-like variants. The important genomic differences underlying this increased risk of CIN 2-3 remain to be determined. [J Natl Cancer Inst 1997;89:796-802]

To date, more than 80 types of human papillomaviruses (HPVs) have been identified and characterized. New HPV types are defined by less than 90% homology with other types when comparing the DNA sequence of the complete L1 coding region; subtypes differ by between 2% and 10%, and variants differ by less than 2% (1). Different types of HPVs vary in tissue affinity and pathogenicity (2). The possibility that the differences in biologic characteristics might also be present among intratypic HPV variants deserves consideration.

Epidemiologic studies (3-6) have demonstrated strong and consistent associations between detection of HPV16 DNA and risk for cervical intraepithelial neoplasm (CIN) and cervical cancer. However, HPV16 is also the most common type of HPV in the cytologically normal population (7), and only a minority of women with HPV16 infection develop cervical cancer (8). Experimental studies have shown that nucleotide alterations of HPV16 affect the potential for oncogenic transformation in vitro (9), and some alterations enhance promoter activities that drive transcription of oncoproteins E6 and E7 (10). Studies (11-14) of genomic heterogeneity of HPV16 demonstrate the presence of multiple variants in all populations examined to date. These observations suggest that natural variants of HPV16 (e.g., prototype-like and nonprototype-like) in a population may not have the same biologic behavior.

The purpose of this study was to evaluate the relationship between natural variants of HPV16 and the risk of biopsy-
confirmed CIN grade 2 or 3 (the most important precancerous lesion of the uterine cervix) (15,16).

Methods

Study Population and Design

Study subjects were from two cohorts: female students enrolled in a university from 1990 through 1995 and women presenting to a sexually transmitted disease clinic from 1989 through 1995. All subjects provided written informed consent to participate in a protocol that had been approved by the University of Washington Institutional Review Board. At the time of the enrollment visit, the university students were all between 18 and 20 years of age. The original cohorts and study design have been described (3,17). Face to face, structured interviews were conducted to elicit information on demographic characteristics, sexual behaviors, and history of sexually transmitted diseases. Vulvovaginal and cervical cell samples for HPV detection and typing were collected at enrollment and approximately every 4 months thereafter. Separate dacron-tipped cervical and vulvovaginal swab specimens were placed in separate vials containing 1 mL of Specimen Transport Medium (Digene Diagnostics, Silver Spring, MD). Samples were prepared for polymerase chain reaction (PCR) assays in a laboratory area separate from that used for PCR product analysis with the use of reagents and equipment dedicated to sample preparation. The PCR amplification was performed by use of HPV1 consensus primers MY09 and MY11 and human B-globin primers PC04 and HG20 (18). Products of PCR amplification were blotted onto filters and hybridized with a generic HPV probe and with mixtures of type-specific oligonucleotide probes including: HPV6 and HPV11; HPV31, HPV33, HPV35, and HPV39; HPV16; HPV18; and HPV45.

Eligible subjects for this study were required to be without CIN 2-3 at entry and to have at least one HPV16-positive visit either at entry or during follow-up. A total of 123 women who met these criteria were included in the analysis. The women included were followed from the date of first HPV16-positive visit to the date of biopsy-confirmed CIN 2-3, loss to follow-up, or the end of 1995, whichever was earliest. Thirty-seven women, including 12 university students and 25 sexually transmitted disease clinic patients, were lost to further follow-up after an average of 4.8 visits (range, 1-14 visits) and were therefore censored at the date of their last visit. Reasons for loss to follow-up included the following: moved out of state (n = 11), unable to locate after three or more attempts (n = 13), refusal (n = 12), and major surgery (n = 1). The number of visits for each subject after the initial detection of HPV16 DNA ranged from 1 to 12, with a mean of 5.7 for women attending the university, and from 1 to 16, with a mean of 7.0 for women presenting to the sexually transmitted disease clinic. During follow-up, subjects found to have lesions that were colposcopically or cytologically suggestive of CIN 2-3 were referred for biopsy. The preparation of biopsy specimens and the staining of slides were standardized (19,20). Histologic diagnoses were assigned as negative, mildly atypical, or consistent with CIN grade 1, 2, or 3 (20) without knowledge of clinical information and HPV status. None of the women had cytologic, colposcopic, or histologic evidence of invasive cervical cancer.

Characterization of HPV16 Variants

Specimens positive for HPV16 in screening were further assayed by PCR-based single-strand conformation polymorphism (SSCP) analysis to identify variants. The assay was conducted as previously described (21). Briefly, DNA amplification was completed in a Perkin-Elmer 9600 Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT) with 35 cycles. Each cycle consisted of denaturation (94°C, 25 seconds), annealing (62°C, 25 seconds), and extension (72°C, 50 seconds). [α-35S]Deoxyadenosine triphosphate (Du Pont NEN, Boston, MA) was incorporated into PCR products during the amplification with a pair of type-specific primers C and D (21), targeting 682 base pairs (bp) from nucleotides 7445 to 222 in the HPV16 noncoding region. PCR products were cleaved into three fragments, 318, 166, and 198 bp from 5’ to 3’, by restriction endonuclease digestion with Dde I, and electrophoresed in a 5% polyacrylamide gel with 10% glycerol. The SSCP patterns of three fragments in the noncoding region were compared individually with the reference patterns from prototype plasmid HPV16 (pHPV16) DNA (22) and HPV16 in DNA of CaSki cervical cancer cells (23) and with the patterns from other specimens. The designation of SSCP patterns was the same as that previously described (21). HPV16 isolates were regarded as different variants as long as any one of the three fragments showed different polymorphisms. On the basis of previous findings of the association between SSCP patterns and sequence variation (24), all HPV16 variants were classified into two groups. The prototype-like group included variants that displayed the reference SSCP pattern and variants with one or two fragments that had only one or two nucleotide alterations. The nonprototype-like group included variants that displayed nonreference SSCP patterns in all three fragments and that had many nucleotide alterations when compared with the HPV16 prototype. The determination and classification of variants was performed without knowledge of the clinical data.

Direct sequencing of PCR products has been described previously (21). In brief, PCR products of the entire noncoding region were generated with a pair of primers A and D. DNAs were purified with a QIAEX II gel extraction kit (Qiagen Inc., Chatsworth, CA). The purified templates (70-100 ng) were sequenced by use of an ABI PRISM Dye Terminator Cycle Sequencing kit (Perkin-Elmer Cetus) based on the protocol recommended by the manufacturer. The sequencing reactions were run on an Applied Biosystems Model 373 A DNA sequencing system (Applied Biosystems, Foster City, CA). Sequences from nucleotide position 7469 to 191 were determined from both directions with two pairs of primers: primers C and F or primers D and E (21). Sequences were analyzed with Sequencer (Gene Codes Corp., Ann Arbor, MI).

A method designated lineage-specific hybridization (24) has been developed to rapidly assign HPV16 variants to the five previously identified phylogenetic lineages of HPV16 (11). Briefly, HPV16 DNAs from 50 specimens from university women were amplified by nested PCR using E6 primers. The amplified products were hybridized to a set of 23 E6 probes that target sequence variations at nucleotide positions 109, 131, 132, 143, 145, 178, 183, 286, 289, 335, 350, 403, and 532. The sequences of the primers and probes used and the conditions of PCR and hybridization were previously described (24).

Statistical Analyses

The Cox proportional hazards model (25) using the statistical software package entitled EGRET (Statistics and Epidemiology Research Corp., Seattle, WA) was used to estimate relative risks (RRs) and confidence intervals (CIs) for CIN 2-3 associated with HPV16 variants, while adjusting for age (<20, ≥21 years), lifetime number of sexual partners (<5, ≥5 partners), HPV16 status at entry (positive or negative), ethnic group (white or nonwhite), and number of visits positive for HPV16 (1-3, ≥4 visits). Kaplan–Meier product limit estimates were used to plot the cumulative proportion of women in whom CIN 2-3 developed, and a logrank test was used to examine the difference in the overall cumulative proportion between women with HPV16 prototype-like and those with nonprototype-like variants. The mean duration at risk per person was examined by Student’s two-sided t test. Kappa value was used to assess a concordance of the classification of HPV16 variants by two detection systems.

Results

Distribution of HPV16 Variants

Overall, 176 of 482 visits from 57 women attending the university and 207 of 585 visits from 66 women presenting to the sexually transmitted disease clinic were positive for HPV16. Prototype-like variants accounted for most of the HPV16 detected (79%) in the women attending the university and 86% in the women presenting to the sexually transmitted disease clinic. Women with HPV16 prototype-like variants had a similar mean duration of follow-up compared with those with a nonprototype-like variant (25.5 versus 18.4 months in university students; P = .17; 27.1 versus 23.4 months in patients presenting to the sexually transmitted disease clinic, P = .62). The distribution of HPV16 variant groups did not vary significantly by age, lifetime number of sexual partners, ethnicity, HPV16 status at entry, number of visits positive for HPV16, or infection with low-, intermediate-, or other high-risk HPV types (data not shown).
Risk of CIN 2-3 in Relation to HPV16 Variants

During follow-up, 32 women with HPV16 infection developed lesions suggestive of CIN 2-3 by cytology or colposcopy and were referred for biopsy. CIN 2-3 was histologically confirmed in nine (15.8%) of 57 HPV16-positive university students and in 10 (15.2%) of 66 HPV16-positive patients presenting to the sexually transmitted disease clinic. Of the 19 CIN 2-3 cases, seven (37%) were CIN 3. Of seven CIN 3 cases, three (43%) were in women infected with nonprototype-like variants, and of 12 CIN 2 cases, five (42%) were in those infected with nonprototype-like variants. Thus, the histologic appearance of biopsy-confirmed lesions was similar for women with nonprototype-like and prototype-like variant infections. Factors including age, lifetime number of sexual partners, HPV16 status at entry, race or ethnicity, and number of visits positive for HPV16 were not significantly associated with risk of biopsy-confirmed CIN 2-3 in either of the two cohorts (Table 1). Among women attending the university, a 6.5-times greater risk (95% CI = 1.6-27.2) of biopsy-confirmed CIN 2-3 was associated with HPV16 nonprototype-like variants as compared with prototype-like variants (Table 2). A similar tendency of increased risk associated with HPV16 nonprototype-like variants was observed among women presenting to the sexually transmitted disease clinic (RR = 4.5; 95% CI = 0.9-23.8). Additional adjustments for low-, intermediate-, and other high-risk HPV types did not substantially change the risk for CIN 2-3 associated with nonprototype-like variants (RR = 7.0; 95% CI = 1.1-44.2 for university students and RR = 4.4; 95% CI = 0.8-23.8 for women presenting to the sexually transmitted disease clinic). When data were restricted to women with HPV16-positive cervical specimens, university women with nonprototype-like variants were 4.2 times (95% CI = 1.0-16.8) more likely to develop biopsy-confirmed CIN 2-3 than those with prototype-like variants. The trend of increased risk associated with nonprototype-like variants was still observed among women presenting to the sexually transmitted disease clinic (RR = 2.7; 95% CI = 0.7-10.7). With the exclusion of women who were positive for HPV16 at entry, risk of CIN 2-3 remained elevated among university students with incident HPV16 nonprototype-like variants relative to those with an incident prototype-like variant infection (RR = 10.1; 95% CI = 1.8-55.8). Risk estimates based on incident HPV16 infection were not available for the cohort of patients presenting to the sexually transmitted disease clinic.

As Fig. 1, A, shows, the overall cumulative proportion of university students who developed CIN 2-3 was higher among those with HPV16 nonprototype-like variants than that among those with prototype-like variants. The overall trend of increased risk among university students with incident HPV16 nonprototype-like variants was still observed among women presenting to the sexually transmitted disease clinic (RR = 2.7; 95% CI = 0.7-10.7). With the exclusion of women who were positive for HPV16 at entry, risk of CIN 2-3 remained elevated among university students with incident HPV16 nonprototype-like variants relative to those with an incident prototype-like variant infection (RR = 10.1; 95% CI = 1.8-55.8). Risk estimates based on incident HPV16 infection were not available for the cohort of patients presenting to the sexually transmitted disease clinic.

Table 1. Characteristics of university students and of patients presenting to the sexually transmitted disease clinic in relation to biopsy-confirmed CIN grade 2-3

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>University students (n = 57)</th>
<th>Patients presenting to the sexually transmitted disease clinic (n = 66)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of subjects</td>
<td>No. with CIN 2-3</td>
</tr>
<tr>
<td>Age at entry, y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤20</td>
<td>57</td>
<td>9</td>
</tr>
<tr>
<td>≥21</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>Racial group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>46</td>
<td>6</td>
</tr>
<tr>
<td>Nonwhite†</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>Lifetime No. of sexual partners at entry</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤5</td>
<td>41</td>
<td>6</td>
</tr>
<tr>
<td>≥6</td>
<td>16</td>
<td>3</td>
</tr>
<tr>
<td>HPV16 status at entry</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>33</td>
<td>6</td>
</tr>
<tr>
<td>Positive</td>
<td>24</td>
<td>3</td>
</tr>
<tr>
<td>No. of visits positive by PCR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-3</td>
<td>37</td>
<td>6</td>
</tr>
<tr>
<td>≥4</td>
<td>20</td>
<td>3</td>
</tr>
</tbody>
</table>

*NA = not applicable; RR = relative risk; CI = confidence interval; CIN = cervical intraepithelial neoplasia; HPV = human papillomavirus; PCR = polymerase chain reaction.
†Nonwhite, including black, Asian, Hispanic, and others.
The 30-month cumulative proportion of university students who developed CIN 2-3 from the time of the first visit positive for HPV16 was 57% for those with nonprototype-like variants as compared with only 13% for those with a prototype-like variant. Among women presenting to the sexually transmitted disease clinic, the 30-month cumulative proportion of women with CIN 2-3 was 39% for those with nonprototype-like variants and 16% for those with prototype-like variants (logrank test; \( P = .01 \)). The 30-month cumulative proportion of university students who developed CIN 2-3 from the time of the first visit positive for HPV16 was 57% for those with nonprototype-like variants as compared with only 13% for those with a prototype-like variant. Among women presenting to the sexually transmitted disease clinic, the 30-month cumulative proportion of women with CIN 2-3 was 39% for those with nonprototype-like variants and 16% for those with prototype-like variants (logrank test; \( P = .10 \) (Fig. 1, B). Of 19 patients with CIN 2-3 from two cohorts, 17 (89%) developed CIN 2-3 within 30 months of the initial detection of HPV16. Two women who presented to the sexually transmitted disease clinic developed CIN 2-3 approximately 40 months after their first positive HPV16 DNA test. Both of these women had an HPV16 prototype-like variant. Six of the 19 women with CIN 2-3 were negative for HPV16 DNA on the visit prior to biopsy. However, only one of the six had a nonprototype-like variant, and the other five had prototype-like variants.

**Characterization of Prototype-Like and Nonprototype-Like Variants**

The correspondence between various genotyping schemes, including SSCP, sequencing, and lineage-specific hybridization, and the resultant nomenclature is shown in Fig. 2. DNA sequencing of HPV16 from nucleotide position 7469 to position 191 was performed in 19 specimens selected to be representative of all variants collected in this investigation. No DNA sequence changes occurred that were not reflected as alterations in the SSCP patterns, except for a T-to-G transition at nucleotide 42 in specimen HHH245. The SSCP pattern of the 198-bp fragment for this specimen could not be distinguished from an A-to-G alteration at nucleotide 131 observed in specimens HHH103 and HHH228 and in the CaSki cell line. All fragments, which had the same nucleotide alterations, displayed identical SSCP patterns. As compared with pHPV16, variants in the nonprototype-like group carried more nucleotide alterations than variants in the prototype-like group.

Sequence variation in the HPV16 E6 region for 50 specimens from university students was further assessed by use of lineage-specific hybridization. Of 10 specimens with HPV16 nonprototype-like variants, six were classified as American-Asian variants, two as African variants, one as a European variant, and one as an Asian variant, a subclass of European lineage (11,13). All of the remaining 40 specimens with HPV16 prototype-like variants were classified as European variants. The concordance of the classification of variants between lineage-specific hybridization and SSCP analysis was high (96% exact agreement; kappa value = 0.86). Women with HPV16 non-European variants were 4.5 times more likely to develop biopsy-confirmed CIN 2-3 compared with women with European variants (95% CI = 1.2-16.8).

**Discussion**

Risk of biopsy-confirmed CIN 2-3 was associated with the infecting HPV16 variant, with nonprototype-like variants associated with the higher risk. One possible explanation for the greater risk of CIN 2-3 associated with HPV16 nonprototype-like
variants is that women infected with HPV16 prototype-like variants were more likely to be lost to follow-up. However, length of follow-up was similar for those with prototype-like and nonprototype-like variants. In addition, women followed until the study end point closely resembled those lost to follow-up before reaching a study end point with respect to age at entry, ethnicity, and lifetime number of sexual partners (data not shown).

Alternatively, the increased risk of CIN 2-3 associated with HPV16 nonprototype-like variants may represent a difference in the biologic behavior of variants. In our data, the excess risk for CIN 2-3 associated with HPV16 nonprototype-like variants was not explained by factors implicated in previous studies (27-29), including high lifetime number of sexual partners, more HPV16-positive visits, nonwhite ethnicity, and infection with other HPV types. The consistency of the association between risk of CIN 2-3 and HPV16 nonprototype-like variants across the two demographically different populations supports the hypothesis that biologic properties for HPV16 variants are not the same. In a similar cohort study of homosexually active men presenting to the Seattle-King County AIDS Prevention Project (Xi LF, Kiviat MB: manuscript in preparation), those with nonprototype-like variants were 3.6 times (95% CI 4.1-11.4) more likely to develop biopsy-confirmed anal intraepithelial neoplasia grade 3 or carcinoma in situ. Additional adjustments did not substantially alter the risk estimate. Furthermore, an elevated risk for CIN 2-3 was observed when the variants were classified by use of different testing systems based on regions other than the noncoding region.

It should be pointed out that both prototype HPV16 and the HPV16 prototype-like variants in the CaSki cell line originated from cancer patients. In view of the fact that the increased risk of CIN 2-3 was associated with HPV16 nonprototype-like variants rather than prototype-like variants, one explanation could be that the lesions associated with nonprototype-like variants are not necessarily precursors of cervical cancer but are more likely to be transient lesions resulting from productive HPV16 infection. Nonprototype-like variants might have an intrinsic growth advantage over prototype-like variants.

It is also possible that HPV16 variants might differ in cellular tropism. The type
of cells may be important in determining the natural history of infection with specific variants through two possible ways. First, some types of cells might be more permissive for viral replication, resulting in an overgrowth of particular variants. The observation of an increased rate of nucleic acid synthesis in reserve cells as compared with those of basal cells of squamous portio epithelium (30) suggests that cellular factors in different types of cells might stimulate the production of virions differently. Again, the lesions could be the direct result of a large amount of virus. Second, lesions derived from different types of cells, although showing similar morphologic abnormalities, might have a variable biologic behavior, resulting in different potentials for progression to cervical cancer. It is well-known that only a portion of women with CIN 2-3, if left untreated, would develop cervical cancer. Thus, it is likely that the histologic classification of CIN 2-3 does not completely reflect biologic potential.

The variable neoplastic potential of HPV16 variants is suggested, in part, by in vitro studies, indicating that nucleotide alterations in the noncoding region of HPV16 enhance promoter activity (10) or alter oncogenic potential in the presence of ras oncogene and hormone (9), and that a change in the E6 region may affect cytotoxic T-lymphocyte responses (31). Some common nucleotide alterations in variants from our population were located in nuclear protein-binding sites (32). However, it is unknown which of the reported nucleotide alterations modify biologic function, leading to a change in oncogenicity in vivo. Furthermore, it has been reported that nucleotide alterations in one region of HPV16 often connect to some changes in other regions (12,13). Thus, the sequence variation identified in the noncoding region may reflect a co-segregation of genetic alterations in other regions, including those not examined here. This linkage makes it feasible to tag HPV16 variants based on a partial sequence rather than a whole genome. However, this linkage also complicates an interpretation in targeting a relationship between a particular nucleotide alteration and the manifestation of phenotype.

Several limitations of this study should be noted. The number of study subjects was small, and thus, the CIs were wide. Because of a limited sample size, we grouped individual variants, based on polymorphisms, into two categories. Thus, it is impossible to detect any differences of risk by individual variants within the group. Nonetheless, this classification would not be expected to give an overestimate, given the assumption that HPV16 nonprototype-like variants might represent a group of variants with an increased risk for CIN 2-3. As stated in the "Methods" section, women who were positive for HPV16 at entry were included in the study. The initial HPV16-positive time for those women was unknown. Bias could be introduced if HPV16 status at entry was associated with a particular variant group. However, when the analysis was restricted to women who had incident HPV16 infection (i.e., HPV16 negative at entry), the association between nonprototype-like variants and CIN 2-3 remained strong. Finally, the data do not rule out the existence of other factors that might be associated both with HPV16 variant groups and with risk for CIN 2-3.

In conclusion, the data from the present study suggest that the risk for CIN 2-3 is not the same for all variants of HPV16 and that nonprototype-like variants confer a greater risk compared with prototype-like variants. The important genomic differences underlying this increased risk of CIN 2-3 remain to be determined.

References

(19) Koss LG. Diagnostic cytology and its histo-


Notes

Supported by Public Health Service grants AI31448, AI38383, AI32917 (National Institute of Allergy and Infectious Diseases), and CA34494 (National Cancer Institute), National Institutes of Health, Department of Health and Human Services.

We thank Claire E. Stevens, Diane Adam, and Kathleen Stine for their clinical work on this project.

Manuscript received November 25, 1996; revised March 25, 1997; accepted April 1, 1997.