Association Between Human Papillomavirus Type and Clonal Status of Cervical Squamous Intraepithelial Lesions

Tjoung Won Park, Ralph M. Richart, Xiao-Wei Sun, Thomas C. Wright, Jr.*

Background: Lesions that are histologically classified as precursors of cervical cancer, which are often referred to as squamous intraepithelial lesions (SILs), represent a heterogeneous clinical entity that can be associated with many different types of human papillomaviruses (HPVs) and have a variable biologic behavior. Approximately one half of low-grade SILs behave as non-neoplastic, productive viral lesions that frequently regress spontaneously, whereas the other half behave as neoplasms and either persist or progress to a histologically higher grade lesion. Identification of biomarkers that reliably differentiate those low-grade SILs with the properties of a non-neoplastic viral infection from those with the properties of neoplasia would allow more rational management decisions to be made. Since monoclonality is a hallmark of neoplasia irrespective of organ site, clonal status might represent one such biomarker. Purpose: To better understand the pathobiology of SILs, we analyzed the clonality of low-grade and high-grade SILs and compared their clonal status with associated HPV type. [J Natl Cancer Inst 1996;88:355-8]

Lesions that are classified as cervical cancer precursors represent a heterogeneous clinical entity that can be associated with many different types of human papillomavirus (HPV) and have a variable biologic behavior (1,2). Lesions that are histologically high-grade are usually associated with the same HPV types as are found in invasive cervical cancers (i.e., HPV types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, or 68) (3-4). Since these histologically high-grade lesions tend to either persist or progress, they are usually treated with ablative or excisional techniques. In contrast, management protocols for women with histologically low-grade lesions vary greatly between institutions and clinical settings. Some clinicians prefer to follow patients with low-grade squamous intraepithelial lesions (SILs) without treatment, whereas others aggressively treat all low-grade SILs with the use of ablative or excisional techniques (5,6). These different approaches to the management of low-grade SILs are explained by the paradoxical biologic behavior of this histopathologic entity. Approximately one half of low-grade SILs behave as non-neoplastic, productive viral infections and frequently regress spontaneously in the absence of therapy (2,7). The other half behave as neoplasms and either persist or progress to a histologically higher grade lesion.

Each year, approximately 1.25 million women in the United States are cytologically diagnosed as having low-grade SILs (5). Identification of biomarkers that could reliably differentiate those low-grade SILs with the properties of a non-neoplastic viral infection from those with the properties of neoplasia would allow more rational management decisions to be made. Since monoclonality is a hallmark of neoplasia irrespective of organ site, clonal status might represent one such biomarker (8-10). A polymerase chain reaction (PCR) based method for determining clonality is now available (11-12). This method uses methylation differences adjacent to a polymorphic region of the androgen receptor gene that is inactivated. To better understand the pathobiology of SILs, we have analyzed the clonality of low-grade and high-grade SILs and compared their clonal status with associated HPV type.

Materials and Methods

Materials

One hundred forty formalin-fixed, paraffin-embedded cervical biopsy and loop electrosurgical-procedure specimens originally diagnosed as SILs were obtained from the pathology archives of Columbia-Presbyterian Medical Center and Kyto Diagnostics, New City, NY. Hematoxylin-eosin-stained slides were reviewed in conference by two gynecologic pathologists who were blinded with respect to clonality and HPV status and who used standard criteria to classify these lesions as low-grade (cervical intraepithelial neoplasia [CIN] 1) or high-grade (CIN2 or 3) SILs (2). The consensus pathologic diagnosis was used for this analysis.

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See “Notes” section following “References.”

Journal of the National Cancer Institute, Vol. 88, No. 6, March 20, 1996 REPORTS 355
For the clonal analysis and HPV DNA determinations, two 4-5-μm sections were cut from each paraffin block and placed on a cleaned glass slide. The loci of SILs were identified by comparing the unstained section with a hematoxylin–eosin-stained tissue section. The abnormal cervical epithelium was then microdissected away from the underlying stroma with the use of an inverted microscope to visualize the dissection of the unstained section. The stroma was saved and used as a polyclonal control tissue for the clonal analysis.

Clonal Analysis

Clonality was assessed in this study with the use of a newly developed, PCR-based method that measures X-chromosome inactivation (11,12). The PCR-based method amplifies a segment of the androgen receptor gene that is located on the X chromosome. This segment of the androgen receptor gene is highly polymorphic because of the presence of a hypervariable trinucleotide repeat that is referred to as a (CAG)n-polymorphism (13). Most individuals have two androgen receptor alleles that can be distinguished on the basis of the size of the amplified DNA product obtained by amplification with PCR primers that flank the hypervariable trinucleotide repeat (Fig. 1). The hypervariable trinucleotide repeat of the androgen receptor gene is located close to Hpa II restriction endonuclease cleavage site that is methylated on the inactive X chromosome but is not methylated on the active X chromosome (Fig. 1). After predigestion with Hpa II, the active (unmethylated) allele is cleaved by Hpa II and cannot be amplified. Therefore, only the inactive (methylated) androgen receptor allele is amplified. This preferential digestion and amplification allows X-chromosome inactivation patterns and clonality to be assessed by comparing the ratio of PCR products observed in Hpa II digested and undigested lesional tissue from the same patient. Since polyclonal tissues are composed of cells with a random X-chromosome inactivation pattern, two amplified DNA products of different sizes are identified after PCR of the Hpa II digested tissue. In contrast, monoclonal (i.e., neoplastic) tissues are composed of a single clone of cells that have an identical X-chromosome inactivation pattern, and only a single amplified DNA product is identified after PCR of the Hpa II-digested tissue.

DNA was extracted from the microdissected paraffin-embedded tissue specimens as previously described (14). The isolated DNA was subsequently digested with 20 U Hpa II restriction endonuclease (New England Biolabs, Inc., Beverly, MA) for 2.5 hours at 37 °C and ethanol precipitated prior to PCR. The PCR reaction contained 60 mM Tris–HCl (pH 9.5), 25 mM (NH₄)₂SO₄, 2.5 mM MgCl₂, 2.5 mM each of deoxynucleotide 5′-triphosphate, 1 U Taq polymerase, 60 pmol of radioactively end-labeled primer 1, and 60 pmol of unlabeled primer 2. Since internal labeling with radioactive nucleotides results in two major bands and several minor bands, primer 1 was radioactively end labeled with [γ-3₂P]ATP and primer 2 was unlabeled for each allele. The amplified PCR products were then separated by electrophoresis through a denaturing 6% urea–polyacrylamide gel. After drying, the gel was exposed to a Kodak XAR film.

Detection of HPV

DNA extracted from the epithelium of all 140 tissue samples was analyzed for HPV DNA with the use of two different PCR methods as previously described. In brief, all samples were analyzed for HPV DNA with previously described conditions and L1 “consensus” primers that amplify the L1 region of more than 25 types of HPV that infect the anogenital tract as well as with E6 “consensus” primers and a PCR–enzyme-linked immunosorbent assay (PCR-ELISA) method that specifically identifies HPV types 16, 18, 31, 33, 35, 39, 45, 56, 58, and 65 (4,15,16).

Statistical Analysis

The association between detection of HPV types 16, 18, 31, 33, 35, 39, 45, 56, 58, or 65 and clonal status was assessed using Fisher’s exact test (two-tailed).

Results

One hundred forty formalin-fixed, paraffin-embedded specimens including 101 low-grade (CIN 1) and 39 high-grade (CIN 2 or 3) SILs were examined for nonrandom X-chromosome inactivation. One hundred four of 140 (74%) specimens were heterozygous with respect to the (CAG)n-polymorphism of the androgen receptor gene (i.e., were classified as “informative”) and therefore could be used for clonal analysis. In these 104 informative cases, a polyclonal pattern was identified by two distinct bands of equal intensity was observed in the stromal control tissue (Fig. 2). Twenty-five (64%) of 39 high-grade SILs were classified as informative and in all 25 (100%) cases, only a single allele was detected after amplification, indicating nonrandom X-chromosome inactivation and a monoclonal origin (Fig. 2). Seventy-nine (78%) of the 101 low-grade SILs were heterozygous with respect to the (CAG)n-polymorphism of the androgen receptor gene and were classified as informative. The other 22 (22%) of the 101 cases were homozygous for the polymorphism. Fifty-four (68%) of the 79 informative cases of low-grade SILs had a monoclonal pattern or nonrandom X-chromosome inactivation (i.e., only a single allele was identified), and 25 (32%) of the 79 cases had a polyclonal pattern (i.e., both alleles were identified).

To determine whether there were specific histopathologic correlates of monoclonality in low-grade SILs, we per-
formed a nonblinded histopathologic review of all low-grade SILs. No specific histologic features could be identified that allowed discrimination between monoclonal and polyclonal low-grade SILs.

All evaluable cases (25 [100%] of 25) of high-grade SILs were determined to be monoclonal (Table 1). Although 54 (68%) of 79 evaluable low-grade SILs were monoclonal, 25 (32%) of 79 low-grade SILs were polyclonal. A strong association was observed between HPV type and clonal status, with a total of 71 (47 low-grade and 24 high-grade) SILs determined to be monoclonal and containing HPV types 16, 18, 31, 33, 35, 39, 45, 56, 58, or 65 (Table 1). Two (5%) of 39 high-grade SILs contained other HPV types that were identified with the use of the L1 “consensus” primers and in three (8%), no HPV DNA was detected. Comparison of the X-chromosome inactivation pattern with HPV status indicated that 24 (96%) of the 25 monoclonal high-grade SILs were associated with HPV types 16, 18, 31, 33, 35, 39, 45, 56, 58, or 65 (Table 1).

HPV types 16, 18, 31, 33, 35, 39, 45, 56, 58, or 65 were identified with the use of the E6 “consensus” primers and the PCR-ELISA technique that identified HPV types 16, 18, 31, 33, 35, 39, 45, 56, 58, and 65 in 34 (87%) of 39 high-grade SILs (Table 1). Two (5%) of 39 high-grade SILs contained other HPV types that were identified with the use of the L1 “consensus” primers and in three (8%), no HPV DNA was detected. Comparison of the X-chromosome inactivation pattern with HPV status indicated that 24 (96%) of the 25 monoclonal high-grade SILs were associated with HPV types 16, 18, 31, 33, 35, 39, 45, 56, 58, or 65 (Table 1).

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In this study, we have examined the clonality of morphologically low-grade and high-grade cervical cancer precursors with the use of a PCR technique. This PCR method detects nonrandom X-chromosome inactivation on the basis of methylation differences adjacent to a polymorphic region of the androgen receptor gene. A previous study (17) analyzed the clonality of cervical cancer precursors by measuring the relative proportions of electrophoretic variants of glucose-6-phosphate dehydrogenase. This study reported that all histologic grades of cervical cancer precursors were monoclonal epithelial proliferations. The finding that cervical cancer precursors were monoclonal provided a theoretical basis for the concept of CIN. This concept hypothesized that all histologic grades of cervical cancer precursors form a biologic continuum (18). Over the last several years, the continuum concept has been reevaluated and replaced by a two-tiered histopathologic classification system for cervical cancer precursors (1,2,19). The newer classification system distinguishes lesions that are morphologically low grade (low-grade SILs) from those that are high-grade (high-grade SILs), but was introduced without a reassessment of the

Table 1. Association of human papillomavirus (HPV) type with clonal status of low-grade and high-grade squamous intraepithelial lesions (SILs)*

<table>
<thead>
<tr>
<th>HPV type</th>
<th>Low-grade SILs (No. of cases)</th>
<th>High-grade SILs (No. of cases)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Monoclonal</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>16, 18, 31, 33, 35, 39, 45, 56, 58, or 65</td>
<td>47</td>
<td>0</td>
</tr>
<tr>
<td>Other HPV types*</td>
<td>2</td>
<td>22†</td>
</tr>
<tr>
<td>Negative for HPV</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Total No. of cases</td>
<td>54</td>
<td>25</td>
</tr>
</tbody>
</table>

*Includes HPV types other than HPV 16, 18, 31, 33, 35, 39, 45, 56, 58, or 65. These samples were positive for HPV DNA with the use of the L1 “consensus” sequence polymerase chain reaction (PCR) but negative for HPV DNA with the use of the E6 “consensus” primers and PCR.

†Twenty-two (92%) of 24 low-grade SILs that contained other HPV types were polyclonal (Fisher’s exact test, two-sided, P<.001, excluding uninformative low-grade SILs).
clonal status of low- and high-grade lesions.

Our results confirm, in part, the finding of the earlier studies that used glucose-6-phosphate dehydrogenase variants to assess nonrandom X-chromosome inactivation, since all morphologically high-grade lesions were monoclonal. However, in contrast to high-grade SIL that is invariably monoclonal, lesions that are morphologically low grade were found to be heterogeneous with respect to their clonality. Some morphologically low-grade lesions are polyclonal and presumably represent a non-neoplastic, virally induced proliferation, whereas other lesions are monoclonal and potentially neoplastic. It is important to note, however, that we could not identify specific morphologic features that correlate with clonal status on an unblinded histopathologic review. However, in contrast to morphology, associated HPV type was an accurate predictor of clonal status. Despite the fact that the E6 "consensus" primer PCR-ELISA method that we used to detect "high oncogenic-risk" or "cancer-associated" HPV types does not identify some of the common HPV types occasionally found in cancers, such as HPV 51, 52, and 68, we still observed a strong association between HPV type and clonal status. Overall, we found that 65% of low-grade SILs were associated with HPV types, such as 16, 18, 31, 33, 35, 39, 45, 56, 58, or 65, which are commonly classified as being "high-oncogenic risk" or "cancer-associated" types (20,21). The proportion of low-grade SILs associated with these "high-oncogenic risk" HPV types in the current study is higher than that reported by some earlier studies, but is similar to what we recently detected in a different population of women with low-grade SILs with the use of another HPV detection method (hybrid capture) (3).

Our findings suggest that the histopathologic entity termed low-grade SIL consists of two different types of lesions that are biologically distinct. One lesion is monoclonal and associated with HPV types 16, 18, 31, 33, 35, 39, 45, 56, 58, or 65. The second type of low-grade SIL is polyclonal and associated with other types of HPV. This may have important implications for the clinical management of low-grade SILs. However, since 68% of low-grade SILs are monoclonal but only approximately 15% progress to high-grade SIL during follow-up, clonal status may not completely predict biologic behavior; additional factors, such as ploidy or human leukocyte antigen type, may also play a role in determining which low-grade lesions progress.

References


Notes

Supported in part by a grant from the Columbia-Presbyterian Cancer Center (T. W. Park). Manuscript received August 1, 1995; revised October 25, 1995; accepted November 30, 1995.

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