Cervical cancer screening has been one of the most successful, but one of the most costly, public health screening programs in the past century. Although the burden of cervical cancer death remains high in developing countries without screening programs, the challenge for continued cervical cancer control in developed countries is to refine screening so that those women who are truly at risk for cervical cancer can be targeted. Current screening programs intensively screen the women least likely to experience cervical cancer. In the current cytologic screening system, human papillomavirus (HPV) testing has proved valuable for triage after an atypical cytologic test to identify which women are likely to benefit from colposcopy. On the other hand HPV testing is not an effective triage test for women with low-grade squamous intraepithelial lesion (LSIL) screening results, because a large percentage of women will be infected with high-risk HPV types but never progress to a precancerous lesion; the lesion often will clear within 2 years of detection. At this time, the clinical recommendation for women with LSIL cytologic condition is to undergo colposcopy and biopsy. This recommendation sends approximately 3% of the US screened population to colposcopy annually; the subsequent prevalence of detected cervical intraepithelial neoplasia (CIN) 2/3 is approximately 15%, which is a very low yield at a high cost. Other HPV-based tests have not produced effective clinical triage for women with LSIL results.

The discordance between the high prevalence of high-risk HPV infections and much lower incidence of cervical cancer indicates that additional factors also play a role in oncogenesis. Specifically, the 3q26 region contains sequences for the RNA component of the human telomerase gene, which serves as a template for telomere addition, which is a potential basis for telomerase-based cell immortalization. A 3q26 gain (3q gain) increases in frequency as the severity of the cytologic specimen approaches cervical cancer. There are relatively infrequent gains in 3q in women with LSIL cytologic findings, but up to 70% of women with high-grade squamous intraepithelial lesions (HSILs) and 100% of women with cervical cancer express 3q gain. Testing for 3q gain has been assessed in small numbers of women with LSIL cytologic findings to predict which women will have CIN 2/3 lesions. The true clinical value of this biomarker, though, is its potential power to indicate which women with LSIL cytologic findings are less likely to progress to CIN 2/3 or cancer and hence can be followed more conservatively.

The objective of this study was to determine the negative predictive value of the
3q26 amplification test for persistence or regression of the LSIL abnormality.

**Materials and Methods**

**Cytologic screening**

A set of cervical cytologic specimens with an index diagnosis of LSIL was collected from 2 independent cytopathology laboratories (B.D., M.M.). The set was identified from 64,693 Papanicolaou smears that were collected in New England between 2001 and 2003. From the 1050 specimens with a diagnosis of LSIL, a set of 54 specimens that (1) were readily accessible, (2) had follow-up cytologic or histologic findings, and (3) had an index cytologic slide available for analysis was selected at random. Five of the 54 cases were excluded because of discordant interpretation by the study cytopathologist and the initial clinical diagnosis. Two cases were excluded because of low cellularity and/or inadequate fluorescence in situ hybridization (FISH) quality after hybridization of the index cytologic slide. The index cytologic slides were deidentified before forwarding for 3q analysis, and no patient clinical information was provided. Because the samples were anonymous and no patient information was provided, patient informed consent and institutional review board approval was not required. The women ranged from 14–67 years in age at the index cytologic finding. We stratified the patients into 3 groups in the following manner: the first group of women had a histologic biopsy within 1 year of the index cytologic finding. The second group of women had a follow-up examination for a corresponding histologic biopsy >1 year from the index cytologic finding. The third group of women had only follow-up cytologic review >1 year after the index cytologic finding. Inclusion criteria for keeping the archival slides in the study were agreement between the clinical cytologic diagnosis and the reviewing cytopathologist for the index LSIL. Each slide had been Papanicolaou-stained. Microscopic fields with cells that met the diagnostic criteria for LSIL were identified and recorded. The index slides were subjected to secondary review, according to the Bethesda criteria, by a board-certified cytopathologist (R.W.) who was blinded to the follow-up cyto logic and histologic specimens. No information was available about treatments received by the women during the follow-up periods.

**Laboratory methods**

Slides were soaked in xylene (Fisher Scientific, Pittsburgh, PA) overnight to remove the coverslips then further destained in fresh xylene for a minimum of 1 hour, in isopropyl alcohol for 30 minutes, and in 100% ethanol for an additional 30 minutes. Slides were hydrated in 85% and 75% ethanol then treated with 1.5% ammonium hydroxide (Acros Organics, Geel, Belgium) in 70% ethanol for 20 minutes. After being rinsed with tap water, slides were treated with an acid alcohol solution for 30 seconds to complete the destaining process.

In preparation for FISH, slides were placed in a sodium citrate solution (Zymed Laboratories Inc, San Francisco, CA) for 30 minutes at 80°C, rinsed in deionized water for 1 minute, then treated with proteinase K solution for 20 minutes at 37°C, followed by a 5-minute wash in phosphate-buffered saline solution. The proteinase treatment was then repeated, followed by fixation in 10% formaldeehyde (Fisher Scientific), 50 mmol/L MgCl2 (Sigma-Aldrich, St. Louis, MO) in phosphate-buffered saline solution for 10 minutes. Finally, the slides were rinsed in 2X saline sodium citrate at room temperature and dehydrated in an alcohol gradient. Each slide was examined under both visible light and a fluorescence microscope to assess background. Slides with high background were treated for an additional 30 seconds with acid alcohol then dehydrated in an alcohol gradient.

Slides with <1000 cells were considered inadequate. Slides were hybridized with a probe for the chromosome 3q26 region, labeled with Spectrum Gold (Abbott Molecular, Des Plaines, IL) and a probe for the centromeric alpha-repeat sequence of chromosome 7, as a control, labeled with Spectrum Aqua (Abbott Molecular). The probe for the 3q26 region comprised a series of bacterial artificial chromosome clones that spanned a 500-kilobase region that contained the human telomerase gene TERC and the gene for the alpha catalytic subunit of phosphatidylinositol 3-kinase. Probe mix in hybridization buffer was added to each slide and covered with a glass coverslip. Coverslips were sealed with rubber cement, and the samples were cohybridized by denaturing at 73°C, for 6 minutes then hybridized at 37°C for 48 hours with a ThermoBrite (Abbott Molecular).

After hybridization, slides were washed for 2 minutes in 2X SSC, 0.3% NP40 (Abbott Molecular) at 73°C, and 2 minutes at room temperature in 2X SSC, 0.1% NP40. The slides were counterstained with 4’,6-diamidino-2-phenylindole (DAPI), dehydrated, and cover slipped with anti fade-containing mounting medium (44 mmol/L 1,4-diazabicyclo[2.2.2]octane [Fisher Scientific]; 89.55% glycerol, 20 mmol/L Tris-HCl, pH 8.0) and placed at –20°C for 15 minutes to enhance signals.

**FIGURE**

Detection of 3q gain in an archival LSIL specimen

3q fluorescence in situ hybridization (FISH) signals are colored gold and control centromeric 7 FISH signals are colored aqua. All 3 cells exhibit more than 4 3q (gold) FISH signals.

Microscopic analysis

The index cytologic slide for each subject was examined by FISH analysis. After hybridization, areas of interest that had been recorded previously were examined with a fluorescent microscope (BX 51; Olympus, Center Valley, PA) equipped with SpectrumGold, SpectrumAqua, and DAPI filters (Abbot Molecular) for nuclei with abnormal DAPI and FISH signals. The number of target nuclei scored and the number of FISH signals for 3q and CEP 7 were enumerated with either a ×60 or ×100 dry objective. Approximately 15–20 fields were examined for each slide.

A case was considered positive for 3q gain by having ≥2 cells with ≥3 3q-FISH signals (Figure).22 Our rationale for this was that a Papanicolaou smear requires a single abnormal cell for a case to be regarded as abnormal; therefore, by requiring 2 abnormal cells, we set a more stringent threshold. We avoided some of the difficulties that have been encountered in previous studies based on the percentage of nuclei with 3 copies of 3q caused, in part, by artifacts overlapping cells and split FISH signals. It also allowed us to exclude likely tetraploid nuclei from the analysis, because the significance of tetraploides is not yet well understood.14 This approach provided for a stringent threshold for determining positivity for 3q gain.

Statistical methods

Sensitivity, specificity, and negative predictive values were calculated by the traditional epidemiologic method with the definition of disease to be either CIN 2/3 or HSIL on the histologic and cytologic endpoints, respectively.23 Nondisease was defined as the persistence of CIN 1 or normal tissue, or the persistence of LSIL or a negative for intraepithelial lesion or malignancy (normal) cytologic finding, for the respective follow-up endpoints. A positive 3q gain test was defined earlier.

RESULTS

Forty-seven index cytologic slides were analyzed for 3q gain. Of the cases studied, 21 women had histologic follow-up examination within 1 year after the index LSIL; 10 women had histologic follow-up examination on average at 21.6 months (± 9 mo; range, 14–45 months) after the index cytologic finding; and 16 women had cytologic follow-up examination on average at 41 months (± 20 mo; range, 18–85 months) after the index cytologic finding.

The results of 3q gain testing on the study population are presented in the Table. In the population of women who underwent biopsy within a year of their index LSIL cytologic finding, the negative predictive value of the 3q gain test was 93% (95% confidence interval [CI], 68–100). For the women who underwent longer-term histology surveillance, on average at 21.6 months, the negative predictive value was 100% (95% CI, 29–100). For those women who underwent the longest surveillance by cytologic finding on average at 41 months, the negative predictive value of the 3q gain test was 100% (95% CI, 74–100).

COMMENT

This retrospective study that used archival specimens indicates that the high negative predictive value of 3q gain for women with LSIL cytologic findings may be able to distinguish which women can be followed conservatively after an LSIL cytologic result. Because <0.5% of women with LSIL truly have CIN 2/3 lesions,11,12 >80% of the women with LSIL may be able to be treated conservatively while their lesion regresses, which would save both the monetary cost and psychologic distress of colposcopic biopsy.

There are limitations to this study, the first of which is the small number of subjects, which necessarily created wide confidence intervals around the negative predictive value point estimate. Even when taken in aggregate for the full 47 women, the confidence interval around the 97% negative predictive value ranged...
from 83–100. Likewise, a possible weakness of this retrospective study of archived specimens is the potential for underrepresentation of the women who progressed to CIN 2/3.

Primary screening for cervical cancer historically has been cytotologic screening, but in the era of HPV vaccination and the recognition of HPV infection as the necessary, but insufficient cause of cervical cancer, new tests for primary screening are centering on some variation of HPV testing. A consensus on test performances for primary screening recommends that the sensitivity and specificity of these new HPV primary screening tests exceed 90%. There is no recommended benchmark, though, for triage testing after abnormal cytologic findings or a positive HPV test for high-risk types. The atypical squamous cells of undetermined significance (ASCUS)–LSIL triage study, that was conducted by the National Cancer Institute showed that the Hybrid Capture 2 test (Digene Corporation, Gaithersburg, MD) that was used for women with an ASCUS result had a negative predictive value of 98.9% for CIN 2/3 within 1 year. Extended data from the atypical squamous cells of undetermined significance/low-grade squamous intraepithelial lesion triage study, that was conducted by the National Cancer Institute showed that the Hybrid Capture 2 test after an initial ASCUS result remains high at 99.7% after 2 years, in part because there is significant regression of untreated CIN 2 lesions within 2 years.

Data from the atypical squamous cells of undetermined significance/low-grade squamous intraepithelial lesion triage study indicates the negative predictive value of the Hybrid Capture 2 test after an initial ASCUS result remains high at 99.7% after 2 years, in part because there is significant regression of untreated CIN 2 lesions within 2 years. Data from the atypical squamous cells of undetermined significance/low-grade squamous intraepithelial lesion triage study refer to a program of primary cytologic findings with secondary triage by high-risk HPV typing. Benchmarks for performance of a triage test in a primary cytologic screening system may reflect the performance of the Hybrid Capture 2 test for ASCUS results. Conversely, effective benchmarks for a test performance for triage of LSIL may be acceptably lower because the annual incidence of LSIL, although significant, is less than ASCUS, and HPV testing does not identify those women who have or will progress to CIN 2/3. Should the primary screening system change to high-risk HPV testing, a secondary triage test would be needed for those women who test positive. The triage test could revert to cytologic screening or could encompass a biomarker, such as a 3q gain, that measures the host’s genetic response to HPV infection.

This study indicates that 3q gain has the potential to triage women with LSIL who are negative for 3q gain to a conservative treatment of the cytologic finding in 1 or 2 years from the index test, while sending those who are positive for 3q gain for colposcopy. Despite limitations, the data presented here for 3q gain in women with an index LSIL cytologic result encompass high negative predictive values that support future prospective clinical trials.

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