Original Article

Prediction of Outcome in Patients With Low-Grade Squamous Intraepithelial Lesions by Fluorescence In Situ Hybridization Analysis of Human Papillomavirus, *TERC,* and *MYC*

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BACKGROUND: Cytology is an excellent method with which to diagnose preinvasive lesions of the uterine cervix, but it suffers from limited specificity for clinically significant lesions. Supplementary methods might predict the natural course of the detected lesions. The objective of the current study was to test whether a multicolor fluorescence in situ hybridization (FISH) assay might help to stratify abnormal results of Papanicolaou tests. **METHODS:** A total of 219 liquid-based cytology specimens of low-grade squamous intraepithelial lesions (LSIL), 49 atypical squamous cells of undetermined significance (ASCUS) specimens, 52 high-grade squamous intraepithelial lesion (HSIL) specimens, and 50 normal samples were assessed by FISH with probes for the human papillomavirus (HPV), *MYC,* and telomerase RNA component (*TERC*). Subtyping of HPV by polymerase chain reaction (PCR) was performed in a subset of cases (n=206). **RESULTS:** There was a significant correlation found between HPV detection by FISH and PCR ($P<.0001$). In patients with LSILs, the presence of HPV detected by FISH was significantly associated with disease progression ($P<.0001$). An increased *MYC* and/or *TERC* gene copy number (>2 signals in>10% of cells) prevailed in 43% of ASCUS specimens and was more frequent in HSIL (85%) than in LSIL (33%) (HSIL vs LSIL: $P<.0001$). Increased *TERC* gene copy number was significantly correlated with progression of LSIL ($P<.01$; odds ratio, 7.44; area under the receiver operating characteristic curve, 0.73; positive predictive value, 0.30; negative predictive value, 0.94) **CONCLUSIONS:** The detection of HPV by FISH analysis is feasible in liquid-based cytology and is significantly correlated with HPV analysis by PCR. The analysis of *TERC* gene copy number may be useful for risk stratification in patients with LSIL. ***Cancer (Cancer Cytopathol)*** **2013;121:423–31.** *© 2013 American Cancer Society.*

KEY WORDS: squamous intraepithelial lesion; human papillomavirus; telomerase RNA component (*TERC*); *MYC*; cervical cytology; fluorescence in situ hybridization.

INTRODUCTION

Cervical cytology has been instrumental in reducing both the incidence and mortality of invasive squamous cell carcinomas of the cervix.[1] However, with the establishment of screening programs, the triage of women with

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low-grade squamous intraepithelial lesions (LSIL) has become an issue because the majority of LSIL cases are likely to regress spontaneously.[2] Even high-grade lesions regress in up to 40% of cases.[3] Therefore, biomarkers that can identify either lesions with a high risk of progression or those with a high probability of spontaneous regression could greatly aid in the management of patients.[4] Infection with high-risk human papillomavirus (HPV) is a necessary but not sufficient precondition for the occurrence of high-grade lesions.[5] Testing for HPV can further help to triage women who have been diagnosed with atypical cells of undetermined significance (ASCUS).[6,7] However, in patients with LSIL, the detection of HPV does not appear to aid in treatment decision-making because the vast majority of these women will test positive for high-risk HPV.[2] Markers especially associated with cell proliferation (eg, Ki-67 or minichromosome maintenance protein 2 (Mcm2)/DNA topoisomerase II-α) were tested in numerous studies for their potential in diagnosing, grading, and predicting the behavior of cervical lesions.[8] An assay combining p16 and Ki-67 has already shown higher specificity than HPV testing for detecting underlying high-grade lesions in women diagnosed with LSIL.[9]

Chromosomal aberrations, which are a hallmark of cancer, have been described in neoplastic squamous lesions of the cervix. Among them are gains of 3q, in which the human telomerase RNA gene (*hTERC*) is located.[10] *hTERC* is the RNA subunit of telomerase that provides telomere stability and regulates telomere length.[11]

TERC appears to be a promising candidate for assessing the malignant potential of preinvasive cervical lesions because an increase in *TERC* gene copy numbers has been shown to be strongly associated with the progression of cervical intraepithelial neoplasia (CIN) to invasive carcinomas.[12,13]

c-MYC is another potential marker for the risk of disease progression in gynecological cytology. *MYC* is involved in a large number of cellular pathways influencing cell proliferation, differentiation, and tumorigenesis.[14] Coamplification of HPV sequences and *MYC* have been demonstrated in cell lines of carcinomas of the lower genital tract.[15] Integration of HPV-DNA appears to occur preferably at specific sites, among them at the locus 8q24, where *c-MYC* is located.[16] An increased copy number (ICN) of *c-MYC* might serve as a biomarker for genetic instability in HPV-infected cells of cervical dysplasia.[17]

The objective of the current study was to assess a multicolor fluorescence in situ hybridization (FISH) assay containing probes for HPV-DNA, 3q26, and 8q24 in liquid-based cervical cytology, especially the capability of this assay to predict the clinical course in patients with LSIL.

MATERIALS AND METHODS

Cytologic Specimens and Patient Data

A total of 425 liquid-based cytology samples of the lower genital tract that were diagnosed between 2007 and 2009 in the cytology division at University Hospital Basel according to the Bethesda classification were included in the study based on adequacy of the material. In a subset of cases (n=206), data regarding HPV status as assessed by polymerase chain reaction (PCR) were available. This PCR assay detected both high-risk and low-risk viruses. Clinical data were collected by reviewing the charts.

For the follow-up data in patients with LSIL, we used the categories of regression, persistence, and progression. Regression was assumed if an LSIL diagnosis was followed by 2 negative Papanicolaou smears and/or negative histology over a period of at least 6 months. Persistence was defined as repeated detection of LSIL over a period of at least 6 months on cytology and/or histology. Progression of LSIL was postulated if LSIL was followed by 2 Pap smears with a diagnosis of high-grade squamous intraepithelial lesions (HSIL) or 1 histological examination with a diagnosis of at least CIN 2 (CIN2).

According to these criteria, there was regression in 67 cases, persistence in 55 cases, and progression in 10 cases of LSIL.

The study was approved by the local ethics committee (Ethical Committee no. 356/07).

FISH for HPV, MYC*, and* TERC

Slides were prepared from samples of liquid-based cytology specimens (BD SurePath liquid-based Papanicolaou test; Becton Dickinson AG, Allschwil, Switzerland), fixed in alcohol, air-dried, and stored at −20°C. Three-color FISH analysis was performed using a probe set targeting *TERC* at chromosome band 3q26 (Vysis LSI *TERC*) and *MYC* at 8q24.2 (Vysis LSI *MYC*), and an HPV probe that detects HPV types 16, 18, 26, 31, 33, 35, 39, 45, 52, 53, 56, 58, 59, 66, and 82.[17,18] The probes were provided by Abbott Molecular, Inc (Des Plaines, Ill) through a

cooperative research and development agreement. The HPV probe was labeled with biotin, *MYC* was labeled with the SpectrumRed fluorophore, and the *TERC* probe was labeled with SpectrumGold. Hybridization procedures were performed according to the protocol provided by the manufacturer.

In brief, slides were incubated for 2 minutes in 2× standard saline citrate (SSC) at 73°C followed by pepsin (0.5 mg/mL in 0.01 N hydrochloric acid) at 37°C for 13 minutes. Slides were fixed in 1% neutral-buffered formalin at room temperature for 5 minutes, and washed in phosphate-buffered saline for 5 minutes. Slides were dehydrated in an ethanol series and air-dried. Co-denaturation at 73°C for 2 minutes and overnight hybridization at 37°C were performed on a HyBrite system (Abbott Molecular). After, the slides were washed in 2× SSC/0.3% Nonidet P-40 (Sigma-Aldrich, St. Louis, Mo) for 2 minutes at 48°C and in 2× SSC/0.1% Nonidet P-40 for 1 minute at room temperature. The HPV probe was detected using the Alexa Fluor 488 Tyramide Signal Amplification Kit 22 (Invitrogen, Carlsbad, Calif) according to the manufacturer's directions. Finally, DAPI (Abbott Molecular) was applied as an antifade solution and slides were coverslipped.

Assessment of FISH

Evaluation of FISH results was performed without knowledge of the cytological diagnosis and clinical data. For assessment of the HPV signals, the entire slide was screened first. If there were HPV-positive cells, 5 high-power fields were evaluated more closely and individual cells were assessed for their HPV signals. The results for HPV were classified as negative or positive with solely diffuse, solely punctuate, or punctuate and diffuse patterns (Fig. 1) for each cell, assessing up to 50 cells in the above-mentioned high-power field. If there were <50 cells on a slide, as many cells as possible were evaluated. The HPV-positive cells marked during the cytological analysis were relocated using the Metafer 4 Scanning System (MetaSystems GmbH, Altlussheim, Germany) to assess *MYC* and *TERC* gene copy numbers. The number of signals for *TERC* and *MYC* were evaluated per nucleus. If the number of HPV-positive cells was <50, cells with abnormal nuclei consistent with dysplasia were preferentially analyzed. Image acquisition and analyses were performed using an Axioplan 2 fluorescence microscope (Carl Zeiss AG, Feldbach, Switzerland) equipped with optical filters (Abbott Molecular Inc) and a × 40 EC Plan-Neofluar (0.75) objective. All slides were assessed by 2 observers. In the case of discrepancies, the results were discussed with a third observer until a consensus was reached.

Statistical Analysis

Analysis of categorical variables and their association with outcome were analyzed using the chi-square or Fisher exact test, where appropriate. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were obtained. Logistic regression analysis was performed and odds ratios (OR) with 95% confidence intervals (95% CI) were obtained to determine the effect of each variable on outcome. The area under the receiver operating characteristic curve (AUROC) was also evaluated to determine the discriminatory ability of each feature (*MYC*, *TERC* and *MYC* and/or *TERC*) for the outcome. Values closer to 1.0 indicate stronger discrimination whereas those approaching 0.5 are the least discriminatory. P values $<.05$ were considered to be statistically significant. Analyses were performed using SAS statistical software (version 9.2; SAS Institute Inc, Cary, NC).

RESULTS

Cytologic Characteristics and Follow-Up Data

A total of 219 LSIL specimens, 52 HSIL specimens, 49 ASCUS specimens, and 50 normal samples of liquid-based cytologies (SurePath) from the uterine cervix were included in the current study. Cases with discrepant results in simultaneous assessment of different samples (eg, ectocervix vs endocervix, cytology vs histology) were excluded. In the LSIL specimens, regression was observed in 67 cases, persistence was noted in 55 cases, and progression was noted in 10 cases. Follow-up data for ASCUS and HSIL were not collected.

Correlation Between PCR and FISH Data for HPV Detection

In 163 cases of ASCUS, LSIL, and HSIL, information regarding the HPV status by PCR was available. Approximately 84% of cases (109 of 130 cases) that were positive for HPV by PCR were also positive by FISH (punctate and/or diffuse staining), whereas approximately 36% of the PCR-negative cases demonstrating signaling on the FISH test (12 of 33 cases). This translated to a sensitivity of 0.84

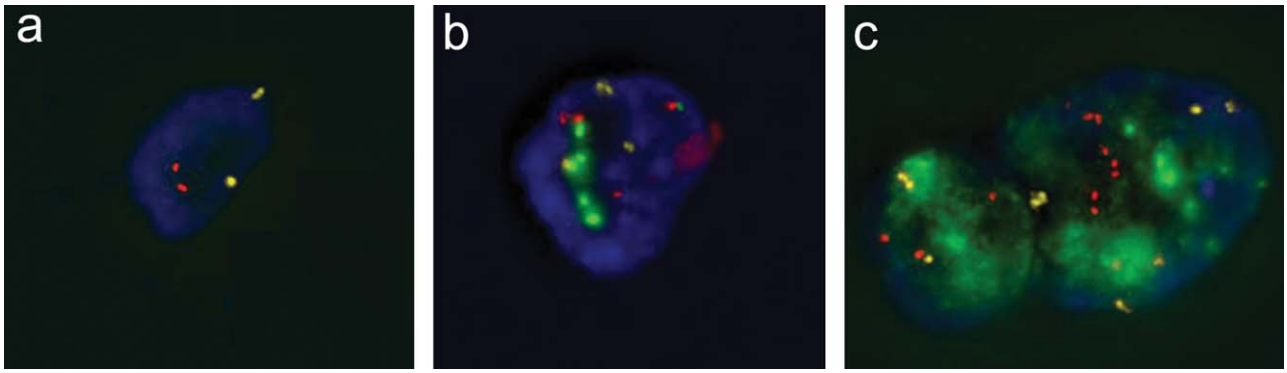

FIGURE 1. Fluorescence in situ hybridization demonstrating human papillomavirus (HPV) (biotin; green), telomerase RNA component (*TERC*) (SpectrumGold), and *MYC* (SpectrumRed) is shown. Nuclei are counterstained with DAPI (blue). (a) An epithelial cell with a small inconspicuous nucleus demonstrated 2 signals for both *TERC* and *MYC*. HPV signals could not be detected. (b) The punctuate green signals suggested integrated HPV-DNA. Increased gene copy numbers were present for both *TERC* and *MYC*. (c) The smaller cell on the right demonstrated a diffuse HPV pattern, suggesting an episomal location. The larger cell on the left contained both punctuate and diffuse signals, suggesting both integrated and episomal HPV. Both *TERC* and *MYC* signals were found to be increased in number in both cells.

for the FISH test for the detection of PCR-positive HPV cases (95% CI, 0.77-0.89), a specificity of 0.64 (95% CI, 0.47-0.78), a PPV of 0.90, and an NPV of 0.5.

Conversely, the sensitivity of PCR for the detection of FISH-positive cases was 0.90 (95% CI, 0.835-0.94) and the specificity was 0.5 (95% CI, 0.36-0.65), with a PPV of 0.84 and an NPV of 0.64.

HPV Detection by FISH

FISH testing for HPV was performed on 49 ASCUS cases, 219 LSIL cases, and 52 HSIL cases. A punctate HPV FISH pattern was found in 69% of cases (41% of ASCUS cases, 70% of LSIL cases, and 85% of HSIL cases), a purely episomal pattern was noted in 10% cases (18% in ASCUS cases, 11% in LSIL cases, and 2% in HSIL cases), and no HPV-specific signal was noted in 21% cases (41% of ASCUS cases, 19% of LSIL cases, and 13% of HSIL cases), as shown in Figure 2.

MYC *and/or* TERC *Detection by FISH*

An ICN of *MYC* or *TERC* was defined as the detection of ≥3 locus-specific signals in at least 10% of cells, similar to the criteria of Alameda et al.[19]

An ICN of *MYC* or *TERC* was observed in 4% or 2% of normal controls. There was a significant difference of ICN between LSIL (*MYC*: 33%; *TERC*: 31%; and *MYC* and/or *TERC*: 33%) and HSIL (*MYC*: 81%; *TERC*: 85%; and *MYC* and/or *TERC*: 85%) ($P<.0001$). In ASCUS samples, an ICN was observed in 35% of *TERC* cases and in 39% of *MYC* cases. ICN of *MYC* and/or *TERC* was noted in 43% of ASCUS specimens.

An overview is given in Table 1.

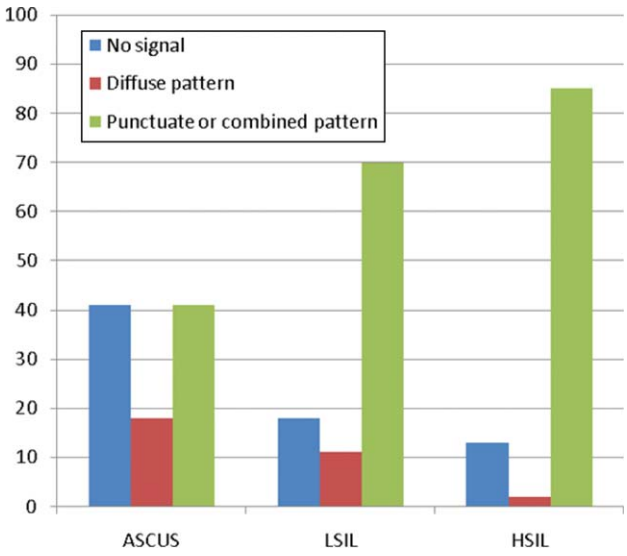

FIGURE 2. The percentages of cases with their respective human papillomavirus signaling patterns are shown. A punctuate pattern or combined (ie, both punctuate and diffuse) pattern was found to be present in 41% of atypical cells of undetermined significance (ASCUS) cases, 70% of low-grade squamous intraepithelial lesion (LSIL) cases, and 85% of high-grade squamous intraepithelial lesion (HSIL) cases. A purely diffuse pattern was found to be present in 18% of ASCUS cases, 11% of LSIL cases, and 2% of HSIL cases; and no HPV specific signals were detected in 41% of ASCUS cases, 19% of LSIL cases, and 13% of HSIL cases. The number of evaluated cases was 49 ASCUS cases, 219 LSIL cases, and 52 HSIL cases.

TABLE 1. Percentages of *MYC*-, *TERC*,- and *MYC*- and/or *TERC*-Positive Cases in Normal Controls, ASCUS, LSIL, and HSIL

Category	No. of Cases	*MYC*-Positive	*TERC*-Positive	*MYC*- and/or *TERC*-Positive
Normal controls	50	4%	2%	4%
ASCUS	49	39%	35%	43%
LSIL	219	33%	31%	33%
HSIL	52	81%	85%	85%

ASCUS, atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesions; HSIL, high-grade squamous intraepithelial lesions; *TERC*, telomerase RNA component.

HPV-FISH and MYC *and/or* TERC *Gains in Relation to Clinical Course in LSIL*

Spontaneous regression was observed in 67 LSIL cases; 73% (n=49) of these were positive by HPV-FISH as opposed to 85% (n=47) of persistent LSIL and all 10 cases of LSIL that progressed to high-grade lesions.

A punctuate or mixed pattern prevailed in 90% of the regressing HPV-FISH–positive LSIL cases, 85% of the persistent HPV-FISH–positive LSIL cases, and all the progressing cases, whereas a purely episomal pattern was detected in 10% of regressing cases and in 15% of persistent lesions that were HPV positive. The difference between a punctuate pattern and a negative/episomal pattern was not found to be statistically significant if regressing LSIL and persistent/progressing LSIL were compared. However, there was a significant correlation noted between the presence of HPV as detected by FISH and regression versus progression in LSIL ($P<.0001$).

The percentage of cases with an ICN was higher for both *MYC* and *TERC* from LSIL specimens with spontaneous regression (*MYC*: 25%; *TERC*: 24%; and *MYC* and/or *TERC*: 25%) over persistent LSIL (*MYC*: 31%; *TERC*: 27%; *MYC* and/or *TERC*: 31%) to LSIL with progression (*MYC*: 60%; *TERC*: 70%; and *MYC* and/or *TERC*: 70%). Comparing regression versus progression, only *TERC* and *TERC* and/or *MYC* ($P<.01$ for both) were statistically significantly different. The sensitivity, specificity, OR, and AUROC for *TERC* were 0.7, 0.76, 7.44 (95%CI, 1.7-32.2), and 0.73, respectively with a PPV and NPV of 0.30 and 0.94, respectively. Similarly for *TERC* and/or *MYC*, the sensitivity, specificity, OR, and AUROC were 0.7, 0.75, 6.86 (95%CI, 1.6-29.6), and 0.72, respectively. The PPV of *TERC* and/or *MYC* for the prediction of progression versus regression was 0.29 and the NPV was 0.94.

Likewise, when cases with persistence and regression were compared with cases with progression, both *TERC* and *TERC* and/or *MYC* indicated a statistically significant increase in their gene copy numbers ($P<.01$ for both). However, both the ORs (*TERC* alone: 6.85, *TERC* and/or *MYC*: 6.04) and the AUROCs (*TERC* alone: 0.72, *TERC* and/or *MYC*: 0.71) were lower than the values for the discrimination of regressing/persisting versus progressing cases.

If only the 111 LSIL cases that were positive for HPV on FISH were considered, there was no statistically significant difference noted between regression and progression for cases with ICN for *TERC*, *MYC,* or *TERC* and/or *MYC*. In the small subgroup of 39 LSIL specimens that were positive for high-risk HPV by PCR, the assessment of the gene copy number of *MYC* or *TERC* did not differentiate between regression and persistence or progression.

DISCUSSION

The management of women with LSIL remains a major challenge. Many of these lesions regress spontaneously whereas only a minority, if left untreated, progress to invasive carcinoma, and prognostic markers are therefore called for.[4] Analysis of HPV only partly aids in the treatment of LSIL because the majority of these lesions are associated with an HPV infection and, more importantly, will regress even if high-risk HPV can be found.[2] In the current study, we found that FISH analysis for copy number gains of *TERC*, a gene involved in the development of cervical squamous cell carcinoma, might be a helpful diagnostic tool with which to better estimate the progression risk of patients with LSIL.

In keeping with previous studies, we found a significant correlation between the detection of HPV by PCR and FISH. Integration of high-risk HPV into the host DNA is considered to be an early key event in cervical carcinogenesis.[20] This is supported by previous reports indicating an association between the status of the HPV infection (ie, replicative state vs integration into the human genome) with the grade of preinvasive lesions and with the risk of progression. Integration of HVP DNA is almost always present in high-grade lesions[21]; however, the reported prevalence of HPV integration varies greatly in the literature, most likely depending on the detection method.[21,22]

In the current study, a punctuate pattern of HPV FISH signals, which has been considered as evidence of viral integration, was similarly common in both LSIL and HSIL (70% and 85%, respectively) and was not found to be associated with disease recurrence or progression. In contrast, the rate of HPV integration in LSIL was considerably lower in previous studies using in situ hybridization technology.[23,24] There are several explanations for this discrepancy: Episomal FISH signals might blur the punctuate pattern. In addition, different detection protocols might contribute to the diverging detection rates of integrated signals between different laboratories.[25] The high prevalence of a punctuate FISH pattern in LSIL noted in the current study would be in keeping with the concept that it is not integrated viral DNA per se but transcriptional activity of integrands after clonal selection that is the most critical step.[20] Alternatively, fluorescent dots might not always represent integrated DNA but normally replicating virus DNA. It has been shown that viruses, including HPV, replicate their genome at compartments or foci rather than randomly within the nucleus.[26] Therefore, the HPV FISH probe might hybridize to the accumulated single-strand DNA of such replication foci, mimicking the punctuate signals noted in the case of integration, thereby explaining the high prevalence of a punctuate pattern (70%) observed in the LSIL cases in the current study. The interpretation of large-sized signals especially may pose a problem for making the distinction between integration sites and replicating foci (Fig. 1).

We conclude that the HPV FISH probe is useful for the detection of HPV-infected cells (eg, to verify PCR results), but does not provide additional information for patient management in general.

The current study data regarding ICN of *TERC* genes in normal samples (2%), LSIL (31%) specimens, and HSIL (85%) specimens were comparable to those in the literature.[27] This is also in keeping with previous reports, in which an ICN of *TERC* genes improved the sensitivity for the detection of underlying high-grade lesions in patients with LSIL.[28,29] According to the recent study by Chen et al, *TERC* analysis showed a higher sensitivity (90%) and specificity (89.6%) than conventional cytology (sensitivity: 84% and specificity: 64.3%) for the detection of ≥CIN2 lesions.[30] Andersson et al[27] were able to distinguish between normal samples/LSIL and HSIL with a sensitivity of 79% and a specificity of 84% by analyzing *TERC*. Statistically significant differences in *TERC*-positive cases were found between LSIL (18.5%) and HSIL (36%) (P=.002) by Kokalj-Vokac et al as well. This so-called stage differentiation (ie, the discrimination between cervical lesions of different grades using *TERC* assessment) has been confirmed in several studies.[12,17,30–33] These previous publications assessing the value of *TERC* assessment in cervical cytology are listed in Table 2.[12,13,17,19,27–33] This table illustrates that the evaluation criteria differ greatly among the studies (eg, the number of evaluated cells may range between 25 cells[32] and 4996 cells[27]). It is certainly a drawback that all these studies used different criteria for *TERC* assessment (eg, different cutoffs), making a comparison of the data difficult.

We defined an ICN in *TERC* as the detection of ≥3 locus-specific signals in at least 10% of cells. To the best of our knowledge, the majority of other studies published to date also have regarded >2 signals as abnormal (Table 2). However, the percentage of cells with an ICN needed to label a case as "positive" varies among studies from between ≥1%[31] to 20%.[13] Our cutoff of 10% is well within this range.

We found more cases with an ICN of *TERC* genes and/or *MYC* alone in the group of LSIL specimens with progression (7 of 10 specimens) than in those with spontaneous regression (17 of 67 specimens), indicating that the assay has prognostic value (P=.0084; sensitivity: 0.7; specificity: 0.75; PPV: 0.29; and NPV: 0.94). The ICN of *TERC* genes alone, but not of *MYC* genes alone, was statistically significantly different between regressing LSIL and progressing LSIL (P=.0062; sensitivity: 0.7; specificity: 0.76; PPV: 0.30; and NPV: 0.94 for *TERC*). The AUROC, which indicates the discriminatory ability of the respective tests (*TERC* and *TERC* and/or *MYC*) for the outcome (regression vs progression) showed fair values (0.73 and 0.72, respectively) for both markers. In contrast, Hesselmeyer-Hadad et al did not detect ICN of *TERC* in cytological samples of CIN1 and CIN2 (n=10) that had regressed spontaneously; however, these cases were only considered positive when >20% of the cells showed a *TERC* signal number of >2. Seven of 12 cases that progressed to CIN3 showed an increased *TERC* gene copy number.[13] Another study concerning *TERC* in 55 LSIL lesions showed a slightly better sensitivity than was noted in the current study for the prediction of persistence/progression in LSIL samples (0.8 at 6 months' control).[19] Taken together, there is mounting evidence that

TABLE 2. Overview of Relevant Studies Assessing the Use of *TERC* Analysis in Cervical Cytology for Stage Differentiation, Prediction of Outcome, and/or Detection of Underlying High-Grade Lesions

Study	No. of Cases	No. and Type of Assessed Cells/Case	Cutoff	Study Endpoint
Heselmeyer-Haddad 2003[31]	57	209-9303 cells	>2 signals in ≥1% of cells	Stage differentiation (normal, ASC, LSIL, HSIL)
Heselmeyer-Haddad 2005[13]	59	Different no. of atypical cells for each case	>2 signals in >20% of cells	Prediction of outcome (progression vs regression)
Hopman 2006[12]	37	100-200 cells	>2 signals in >10%-20% of cells	Stage differentiation (CIN2/CIN3, CIN3/microinvasive carcinoma, invasive carcinoma)
Sokolova 2007[17]	235	≤100 HPV-positive cells	>2 signals per cell	Stage differentiation (normal, CIN1, CIN2, CIN3, carcinoma)
Caraway 2008[32]	66	25 atypical cells	>2 signals per cell	Stage differentiation (normal/ASCUS vs HSIL/carcinoma)
Alameda 2009[19]	55	≥400 cells	>2 signals in >1.6% of cells	Prediction of outcome (progression/persistence vs regression)
Andersson 2009[27]	78	232-4996 cells	>2 signals in >8 cells (excluding tetraploid cells)	Stage differentiation (LSIL vs HSIL)
Kokalj-Vokac 2009	102	30 atypical cells	>2 signals in >4 cells	Stage differentiation (LSIL vs HSIL)
Tu 2009[33]	1033	100 cells	>2 signals in >5.2%, 5.6%, or 6.4% of cells	Stage differentiation (normal, ASCUS, LSIL, ASC-H, HSIL)
Jiang 2010[28]	7786	≥100 cells	>2 signals in >6.4% of cells	Detection of underlying ≥CIN2 in LSIL and ASCUS
Chen 2012[30]	299	≥100 cells	>2 signals in >5% of cells	Stage differentiation (normal/CIN1 vs CIN2/CIN3/carcinoma)
Heitmann 2012[29]	65	<800 cells	>4 signals in ≥2 cells	Detection of underlying ≥CIN2 in LSIL

Abbreviations: ASC, atypical squamous cells; ASC-H, atypical squamous cells cannot rule out a high-grade lesion; ASCUS, atypical squamous cells of undetermined significance; CIN, cervical intraepithelial lesion; HPV, human papillomavirus; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion; *TERC*, telomerase RNA component.

ICN of *TERC* genes might serve as a prognostic marker in LSIL, whereas *MYC* analysis has no added value. In our collective, ICN of *TERC* and *MYC* were almost always simultaneously found in the same cases; in 1 case with progression, the *TERC* signal number was increased but not the *MYC* signal number. Conversely, gene copy numbers for *MYC* but not for *TERC* were found to be increased in 1 regressing and 2 persistent LSIL specimens. Therefore, the assessment of *MYC* diminished rather than enhanced the predictive value of the test assay.

Collectively, our results and those of previous studies suggest that *TERC* gene copy number is a promising marker in LSIL, whereas *MYC* gene copy number may have no major added value.[27,30] There currently are 3 commercial tests for determining *TERC* gene copy number using different probe combinations, technical platforms, and thresholds (Vysis LSI *TERC* SpectrumGold Probe, Abbott Molecular, Inc; CERVIXCYTE, Cancer Genetics, Inc, Rutherford, NJ; and oncoFISH cervical; Ikonisys, Inc, New Haven, CT).

In addition, the definition of clinical endpoints is crucial in assessing the biological behavior. To avoid false-negative results due to sampling errors, we demanded 2 consecutive (instead of 1) negative Pap smears and/or negative histology before regression of LSIL was postulated. Colposcopy was routinely performed in patients with previous abnormal findings in addition to cytology, which minimized the risk of sampling errors.

We defined repeated detection of LSIL over a period of at least 6 months on cytology and/or histology as persistence; however, it might be argued that longer periods of time (eg, up to 2 years) are required to label an HPV infection as persistent; for example, HPV type 16 persists for a mean of approximately 18 months.[34] Furthermore, sampling errors or interpretation errors may lead to false-negative results and an incorrect classification of outcome.

One of the general obstacles in establishing markers for the prediction of the clinical course of cervical lesions is the variability of these lesions, which might be bidirectional.[35] LSIL may first progress to HSIL, but then spontaneously regress again. Therefore, it is important to precisely detect the "point of no return" at which a neoplastic lesion can no longer regress spontaneously. It is clear that high-risk HPV infection is a precondition for the development of invasive cancer, but most infections will clear spontaneously, which leads to a rather low specificity and PPV for HPV testing in primary screening.[36] Further studies are needed for the subgroup of LSIL cases that are positive for high-risk HPV to explore the relative

amount of information gained at the time of prognosis by FISH and by HPV typing.

The authors of an in vitro study using the HPV type 33-positive cell line UT-DEC-1 identified 3 distinct time points that are important for the transformation progress: 1) viral integration and episome loss; 2) the selection of cells with the ability to maintain telomerase activity; and 3) permanent upregulation of telomerase. A marker panel consisting of *hTERT*, Bcl-2, and S100A9 was suggested to be of value for the assessment of CIN.[37] It appears to be probable that the "point of no return" on the way to cervical cancer cannot be defined by a singular molecular event, but rather by a combination of genetic and/or epigenetic parameters given the broad variety of molecular changes that can be induced by HPV infections.[38] However, it is likely that chromosomal instability as indicated by ICN of genes is closer to cancer than preceding transcriptionally active HPV integration and deregulated cell cycle control.

Using a large number of liquid-based cytology samples, we demonstrated that the application and assessment of a FISH assay combining probes for HPV, *TERC*, and *MYC* is feasible, and that *TERC* gene copy number holds the most promise as a prognostic marker for risk stratification in cases of LSIL. Although the punctuate pattern of HPV signals is unlikely to specifically represent HPV integration, HPV FISH helps by flagging cells for the targeted analysis of HPV-infected cells for gene copy number aberrations. Further studies are needed to investigate the molecular mechanisms of HPV-induced carcinogenesis in conjunction with large-scale clinical studies with long-term follow-up to predict the clinical course.

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