

# Clinical Role of the Detection of Human Telomerase RNA Component Gene Amplification by Fluorescence in situ Hybridization on Liquid-Based Cervical Samples: Comparison with Human Papillomavirus-DNA Testing and Histopathology

Roberta Zappacosta<sup>a,b</sup> Manuel Maria Ianieri<sup>c</sup> Danilo Buca<sup>c</sup> Elena Repetti<sup>d</sup>  
Alessandra Ricciardulli<sup>c</sup> Marco Liberati<sup>c</sup>

<sup>a</sup>Surgical Pathology Unit, 'SS Annunziata Hospital' of Chieti, and <sup>b</sup>School of Clinical Biochemistry and  
<sup>c</sup>Obstetric and Gynaecology Unit, 'G. d'Annunzio' University of Chieti-Pescara, Chieti Scalo, and  
<sup>d</sup>Italian Diagnostic Center, Milan, Italy

## Key Words

Cervical cancer prevention · 3q26 chromosome ·  
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## Abstract

**Objective:** This study was designed to evaluate whether the adjunct of human telomerase RNA component (hTERC) fluorescence in situ hybridization (FISH) analysis to cytological diagnosis and human papillomavirus (HPV)-DNA testing may serve as a predictive marker for distinguishing cervical lesions destined to regress from those at high risk of progression towards invasive cancer. **Study Design:** hTERC FISH analysis was performed on 54 residual liquid-based cytology specimens obtained from women referred to colposcopy for the detection of atypical squamous cells of undetermined significance or worse (ASCUS+) lesions. Histological diagnosis was considered the gold standard and cervical intraepithelial neoplasia of grade 2 or worse (CIN2+) as the worst outcome. **Results:** Oncogenic HPV-DNA was found in 96.3% of the specimens. Among these, 38.5% revealed a CIN2+ diagnosis. hTERC gene amplification was detected in 37% of

the cases; among these, 70% showed up as CIN2+. hTERC FISH analysis significantly improves the specificity and positive predictive value of HPV-DNA testing, thus differentiating patients with a CIN2+ diagnosis from those with a CIN2– diagnosis. **Conclusions:** Despite the limitation of a small study sample, our findings provide promising data, indicating the possible role of hTERC analysis in the assessment of the risk of developing cervical cancer. This approach would implement the specificity of DNA testing, avoiding overtreatment at the same time. Prospective follow-up studies are needed with the aim of introducing hTERC FISH into decision-making algorithms.

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Cervical squamous-cell carcinoma (CC) is the second-most common cancer in women worldwide, with at least 400,000 new cases detected every year [1].

Despite being the first neoplasm for which there is an easy preventive and therapeutic approach, CC remains an important cause of morbidity and mortality. This is main-

R.Z. and M.M.I. contributed equally to this paper.

ly due to the low sensitivity and poor reproducibility of cytological diagnosis.

The accuracy of the Papanicolaou (Pap) test has been recently improved by the use of liquid-based cytology (LBC), but its diagnostic performance remains lower, especially for certain diagnostic categories such as atypical squamous cells of undetermined significance (ASCUS) and low-grade squamous intraepithelial lesions (LSIL) [2]. As a consequence, the management of women with such lesions still remains controversial [2].

Epidemiological and molecular studies demonstrated that oncogenic human papillomavirus (HPV) infection represents the most significant risk factor for cervical carcinogenesis. CC in the absence of oncogenic HPV is rare [3]. On the other hand, infection with oncogenic HPV types is a necessary but not sufficient condition.

CC emerges via a series of precursor lesions with an increased degree of severity, classified as cervical intraepithelial neoplasia (CIN) grades 1–3. These lesions retain the capacity to spontaneously regress. The Centers for Disease Control (CDC) calculated that at least 80% of women will acquire HPV infection during their lives, but only 10% of these will develop a persistent infection and be at a high risk of progression towards invasive cancer [4].

The introduction of HPV-DNA testing into the diagnostic algorithm of patients with cervical lesions has certainly improved the sensitivity of the Pap test, but the main problem remains, i.e. the high prevalence of HPV infection in comparison with the low number of women developing invasive cancer [5].

The current concept of CC prevention aims to identify patients who would benefit from medical interventions, without concerning individuals who would not benefit. Currently, it is possible to differentiate 3 levels of risk of developing cervical cancer: (1) infection with oncogenic HPV types, (2) the emergence of cell clones with a deregulated expression of viral oncogenes and (3) the progression of a few mutated clones towards a large cell population, and the subsequent increased risk of chromosomal instability [6]. Thus, the ideal predictive biomarker would have the capacity to early identify cell populations which have acquired chromosomal instability and are able to evade the antioncogene host mechanisms [5].

Integration of the HPV genome into the host genome is certainly a key factor leading to CIN progression. There is consensus that integration is common in high-grade CIN and cancer, but infrequent or lacking in regressing lesions. HPV integration, escaping immune system surveillance, would induce the overexpression of E6 and E7

oncogenes with a consequent accumulation of genetic aberrations [7]. Detection of viral messenger (m)RNA transcripts of E6 and E7 oncoproteins has been found to be a promising approach for the management of women with cervical lesions [7]. Currently, the E6/E7 mRNA test represents the most hopeful test for the identification of cervical lesions with a high risk of progression. However, RNA extraction requires trained human resources and is too labor-intensive for routine application [7].

The deregulated expression of viral E6/E7 oncogenes induces chromosomal instability, resulting in the loss and gain of chromosomal material within the affected cells [8]. One of the most prevalent genomic alterations in HPV-transformed cells involves the long arm of chromosome 3 (3q), which contains the human telomerase RNA component (hTERC) [9]. Telomerases, located at the ends of chromosomes, influence cell replication. They shorten during each cell division and seem to be like a clock for cells [10].

Telomerase consists of two essential components: one is the functional RNA component (in humans called hTERC), which serves as a template for telomeric DNA synthesis; the other is a catalytic protein (hTERT) with reverse transcriptase activity. This complex synthesizes telomeres and stabilizes their length. Mutation in any of these components may result in neoplasia. Telomerase activity is reported to be low in normal human somatic cells [11]. Proliferating cells become senescent when telomeres are shortened or when there is no telomerase activity [11]. Cancer cells overcoming this senescence pathway are immortalized [12]. Immortalization is usually accompanied by abnormal activation of telomerases, which seem to be essential for the progression of neoplastic clones. In view of this, telomerase activity can be used to distinguish between malignant and normal cells [10–12].

The hTERC gene seems to be crucial for the reactivation of telomerases as well as neoplastic transformation by blocking cell apoptosis [12]. For these reasons, hTERC gene amplification has been recently regarded as a marker for predicting prognosis for women with cervical lesions.

This study was designed to determine the correlation between hTERC gene amplification and oncogenic HPV infection, and to assess the potential usefulness of this genomic marker for identifying cervical lesions which are more likely to persist and induce invasive cancer. To do this, this fluorescence in situ hybridization (FISH) technique was used to retrospectively detect the hTERC on exfoliated cells collected from the cervix on behalf of the regional Cervical Cancer Screening Program.

## Material and Methods

### Selection of Patients

This study was performed according to the principles outlined in the Declaration of Helsinki of 1975, and it was approved by the local editorial board.

Case series were extracted from the electronic database of the outpatient department of 'ASL 2 Abruzzo', by analyzing the clinical history of patients who underwent colposcopy and colposcopy-directed biopsy from January 2012 to July 2013. We selected patients referred to colposcopy for ASCUS+ cytological diagnosis. An additional inclusion criterion was the availability (for each patient) of a residual liquid-based cervical specimen collected for cytological diagnosis.

Exclusion criteria were: treatment for cervical lesion in the past 5 years, a history of any type of cancer, surgical or ablative treatment (except for biopsy) during baseline colposcopy, hysterectomy, HIV positivity or other causes of immunodeficiency and pregnancy.

Written informed consent was obtained from the patients and an identification code was assigned to each case, upholding standards of confidentiality.

The LBC result was reported according to the 2001 revision of the Bethesda System [13], as ASCUS, LSIL, high-grade squamous intraepithelial lesion (HSIL) and CC.

Histological diagnosis was classified as normal or benign cellular changes (NILM), CIN1–3 and invasive squamous-cell carcinoma, according to the 2005 WHO guidelines [14]. Two independent surgical pathologists, separately and blinded to all other study results, revised tissue slides and established the final diagnosis, basing it on the highest CIN grade detected on each sample.

We assumed that both the colposcopy and biopsy were 100% sensitive. In view of this, histological diagnosis from colposcopy-directed biopsies was accepted as a verification of disease status and regarded as the gold standard. NILM and CIN1 are referred to here as less severe than CIN2 (CIN2–), and CIN2, CIN3 and CC as CIN2+. CIN2+ was regarded as the worst outcome.

Only patients for whom there was consensus about the histological diagnosis were finally included in the study.

### LBC and HPV-DNA Test

Cervicovaginal samples were collected from the ecto- and endocervix with an Ayre spatula and a Cytobrush. Cytological specimens were then directly transferred in ThinPrep vials (ThinPrep®, Hologic, Marlborough, Mass., USA) containing 20 ml of Preserv-Cyt solution.

To prepare cytological slides from liquid-based cervical samples, a ThinPrep 5000 processor (Hologic Corp., Boxborough, Mass., USA) was used. Slides were then stained with a Pap procedure.

An aliquot (4 ml) of each LBC sample, stored at room temperature, was removed for the detection of oncogenic HPV-DNA by the Digene Hybrid Capture 2 method (HC2, Qiagen, Gaithersburg, Md., USA). A total of 13 oncogenic HPV types were detected (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68). The test was performed according to the manufacturer's instructions. HC2 reactions, as chemiluminescent signals, were then read by the offline luminometer system (Digene microplate luminometer, DML 2000), which provided relative quantification of each sample in comparison to the mean of a series of positive controls containing

1 pg/ml of HPV-DNA (corresponding to approx. 100,000 HPV-16 genomes/ml or 5,000 HPV copies/reaction). A cut-off value of 1 relative light unit, corresponding to >1 pg/ml of HPV-DNA, was used to classify the specimen as positive.

### Amplification of hTERT Gene by FISH

In the laboratories of the Italian Diagnostic Center of Milan, residual liquid-based cervical samples were also used to perform hTERT FISH analysis (Kreatech Diagnostics, Amsterdam, The Netherlands). FISH probes containing the hTERT gene at locus 3q26.2 in spectrum yellow and a control probe at chromosome 7 in spectrum blue were applied.

Cervical cells, sorted and adherent to a glass slide, were subjected to a temperature of 70°C for 5 min to denature the DNA molecules. A specific probe for the 3q26 region, conjugate with yellow dye gold, was applied on each slide. Hybridization with a hTERT (3q26) probe was carried out at 37°C for 14 h. After hybridization, slides were washed with the first post-hybridization wash buffer, at 73°C for 2 min, and with the second post-hybridization wash buffer at room temperature for 1 min.

In order to contrast the yellow gold, nuclear chromatin was stained with DAPI blue dye. Upon completion of the bond between the probe and its complementary region, the chromosome was labeled and highlighted by the binding fluorescence.

Evaluation of the hTERT FISH results was performed blinded to the cytological and histological diagnosis and clinical data. Slides were viewed at ×1,000 magnification on a fluorescence microscope equipped with Spectrum Gold and DAPI filters, by 2 independent observers. In the case of discrepancies, the results were discussed with a third observer until a consensus was reached.

The dark background of the nucleus of the cells appeared as blue due to DAPI staining of the chromatin and the hTERT gene locus appeared as yellow gold. The number of signals for hTERT and chromosome 7 were evaluated per nucleus. In normal cells, each nucleus contains only 2 copies of the telomerase gene (corresponding to 2 yellow spots) and 2 of chromosome 7 (corresponding to 2 blue spots). Thus, the expected ratio in the normal cells was 2:2. Cells with the telomerase gene amplification would contain multiple copies of the 3q26 gene.

In this study and for each cytological specimen, 2,000 cells were scored for an abnormal signal of both 3q26 and centromeric chromosome 7. To avoid counting split signals as 2 signals, the distance between any 2 signals had to be at least the diameter of 1 signal, in order for them to be counted as individual signals. A case was considered positive for 3q26 amplification by having ≥5 locus-specific signals in at least 10% of the abnormal cells according to the criteria of Obermann et al. [15]. Image acquisition and analysis were performed using Ikoniscope digital microscopy system automation (Konisys Inc., New Haven, Conn., USA) equipped with optical filters.

### Statistical Analysis

We calculated the prevalence of HPV-DNA and hTERT FISH positivity using standard methods.

In order to correlate HPV-DNA and FISH results with histological diagnosis, 2 × 2 tables were used. Sensitivity and specificity of each test individually as well as in comparison to one another were assessed by receiver operating characteristic (ROC) analysis, with the histological diagnosis as the gold standard. Areas under the ROC curves with 95% confidence intervals (CIs) were estimat-

**Table 1.** Summary of results from cytological and histological diagnosis, HPV-DNA testing and hTERT FISH analysis

Cytological diagnosis	Histological diagnosis				hTERT FISH result		HPV-DNA test	
	NILM	CIN1	CIN2	CIN3	positive	negative	positive	negative
ASCUS	5 (62.5)	9 (34.6)	7 (77.8)	2 (18.2)	8 (40)	15 (44.1)	21 (40.4)	2 (100)
LSIL	3 (37.5)	17 (65.4)	2 (22.2)	3 (27.3)	7 (35)	18 (53)	25 (48.1)	0
HSIL	0	0	0	6 (54.5)	5 (25)	1 (2.9)	6 (11.5)	0
All	8	26	9	11	20	34	52	2

Values are expressed as n (%).

ed to assess differences in performance, and the McNemar test was used for statistical significance. Positive and negative predictive values (PPV and NPV) were also calculated, and the results were given with 95% CIs.

The agreement of the HPV-DNA test and FISH analysis with the histological diagnosis was calculated by kappa statistics. According to the criteria of Landis and Koch [16], the  $\kappa$  values were divided into 6 scales of strength of agreement: poor (<0.00), slight (0.00–0.20), fair (0.21–0.40), moderate (0.41–0.60), substantial (0.61–0.80) or almost perfect (0.81–1.00) [16].

Odds ratios (ORs) were used to correlate both the HPV-DNA and FISH results with the histological outcome. All statistical analyses were performed with SPSS software v15.0 (SPSS for Windows, SPSS Inc., Chicago, Ill., USA). In all analyses,  $p < 0.05$  was regarded as significant.

## Results

The study included 54 patients (mean age  $35.7 \pm 10.8$ , median 34 and range 22–65 years). Table 1 shows the distribution of patients according to the cytological and histological diagnoses, the HPV-DNA test and the hTERT analysis.

### *hTERT and Cytological Categories*

We detected 23 ASCUS (42.6%), 25 LSIL (46.3%) and 6 HSIL (11.1%) cases. The PPV of the different cytological categories was 39.1% for ASCUS, 20% for LSIL and 100% for HSIL, respectively. The corresponding NPV was 60.9, 80 and 100%, respectively ( $p < 0.05$ ). hTERT FISH showed a clear signal in 100% of the specimens (fig. 1). hTERT gene amplification was found in 37% (20/54) of the cases.

In the ASCUS group, a positive result was detected on FISH in 34.8% (8/23) of the cases. Among these, 75% (6/8) were CIN2+ and 25% (2/8) were CIN2– ( $p < 0.0001$ ). In the LSIL category, hTERT gene amplification was detected in 28% (7/25) of the cases. Among these, 42.9%

(3/7) were CIN2+ and 57.1% (4/7) were CIN2– ( $p = 0.2$ ). Of the HSIL cases, 83.3% showed hTERT gene amplification and all of these were CIN2+ (CIN3).

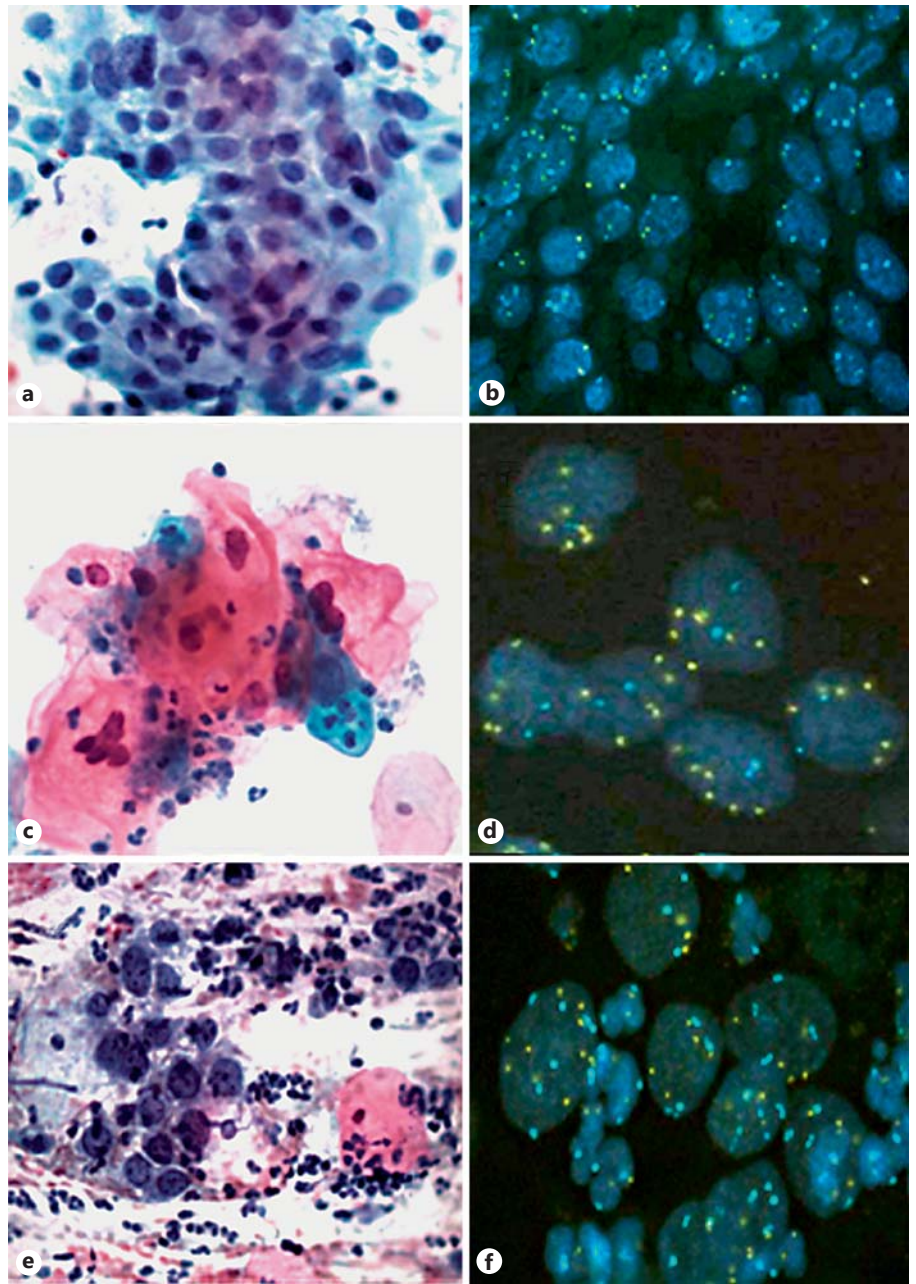
Overall, the mean number of cells with hTERT gene amplification was 45.8 (range 2–500). According to the cytological categories, a mean number of 47.5 cells was detected in the ASCUS category, 67.6 in LSIL and 39.6 in HSIL, but the difference did not reach statistical significance ( $\chi^2$  test;  $p = 0.22$ ).

Table 2 correlates the positive hTERT result with the cytological and histological diagnoses. Within the ASCUS category, hTERT analysis differentiated patients histologically diagnosed as CIN2+ (66.7%) from those diagnosed as CIN2– (14.3%) ( $p < 0.0001$ ). Likewise, hTERT gene amplification differentiated LSIL resulting as CIN2+ (60%) from those revealing CIN2– (20%) ( $p < 0.0001$ ).

The sensitivity, specificity, PPV and NPV of the hTERT analysis, with CIN2+ as the gold standard, were 66.7, 85.7, 75 and 80% for ASCUS, and 60, 80, 42.9 and 88.9% for LSIL, respectively. The sensitivity and PPV for HSIL were 75 and 100%, respectively. No CIN2– were detected in the HSIL group. hTERT FISH analysis significantly improved the PPV of the ASCUS diagnosis ( $p < 0.01$ ), but for the LSIL and HSIL categories, the difference did not reach statistical significance.

### *Correlation between hTERT and Histological Diagnosis*

There were 8 patients with NILM (14.8%), 26 CIN1 cases (48.1%), 9 CIN2 cases (16.7%) and 11 CIN3 cases (20.4). No SCC was detected. The overall CIN2+ prevalence was 37% (20/54 cases); CIN2+ was detected in 39.1% (9/23) of the ASCUS cases, 20% (5/25) of the LSIL cases and 100% (6/6) of the HSIL cases. Among the positive hTERT cases, 70% (14/20) were CIN2+ and 30% (6/20)

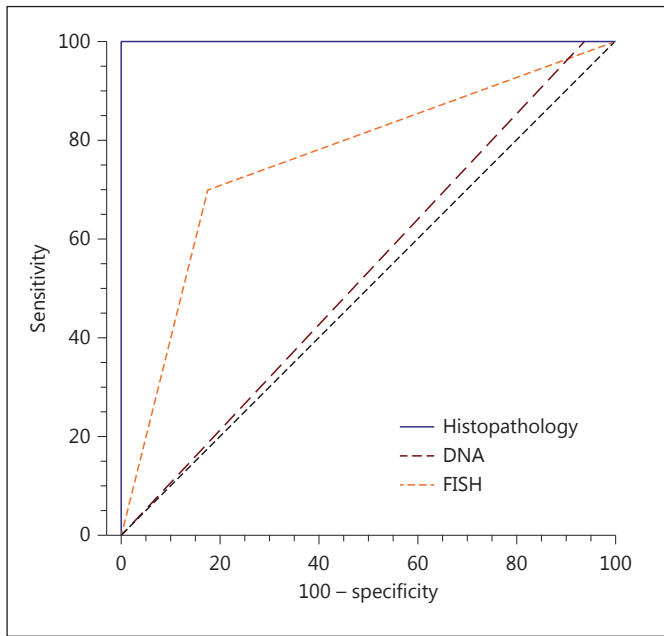


**Fig. 1.** Thin-layer slides from liquid-based specimens. Pap.  $\times 20$  and corresponding hTERC dual-color FISH,  $\times 20$ . hTERC gene = yellow fluorescence and chromosome 7 = blue fluorescence. **a** ASCUS. **b** Normal 2:2 hTERC signal pattern. **c** LSIL. **d** Abnormal 6:2 hTERC signal pattern. **e** HSIL. **f** Abnormal 5:4 hTERC signal pattern.

were CIN2-. Among the negative FISH cases, 82.4% (28/34) showed CIN2- and 17.6% CIN2+ ( $p < 0.05$ ).

Upon considering the histological categories as distinct, no NILM showed FISH amplification, while 23.1% (6/26) with CIN1, 66.7% (6/9) with CIN2 and 72.7% (8/11) with CIN3 tested as hTERC positive. The proportion of hTERC gene amplification significantly increased from CIN1 to CIN3 (Cochran-Armitage test for trend;  $p < 0.0001$ ).

The percent agreement between the FISH results and the histological diagnosis was 77.8% (Cohen's  $\kappa$  value: 0.52). The sensitivity, specificity, PPV and NPV of hTERC FISH were 70, 82.4, 70 and 82.3%, respectively. A positive FISH result was associated with a CIN2+ diagnosis (OR 2.18, 95% CI 1.11–4.28;  $p = 0.02$ ). These data indicate the significant association between hTERC gene amplification and a CIN2+ diagnosis.



**Fig. 2.** ROC curves comparing the sensitivity and specificity of HPV-DNA and hTERC FISH with the corresponding parameters of histological diagnosis ( $p = 0.04$ ). Bisector line indicates a reference threshold with an AUC of 0.5.

### *hTERC and HPV-DNA Test*

HPV-DNA testing showed a positive result in 52/54 (96.3%) of the cases, particularly in 21/23 (91.3%) of ASCUS, 25/25 (100%) of LSIL and 6/6 (100%) of HSIL cases. The proportion of DNA-positive results increased with the severity of cytological lesion (Cochran-Armitage test for trend;  $p = 0.13$ ).

All (20/20) of the CIN2+ and 94.1% (32/34) of the CIN2- patients revealed a HPV-DNA-positive result. Moreover, 87.5% of NILM resulted as DNA positive. A positive DNA test result conferred an OR for CIN2+ of 0.63 (95% CI 0.04–10.57;  $p = 0.7$ ). Overall percent agreement between DNA testing and histological diagnosis was 40.7% (Cohen's  $\kappa$  value: 0.04).

The overall sensitivity, specificity, PPV and NPV for DNA testing were 100% (95% CI 83.8–100), 5.9% (95% CI 1.63–19.1), 38.5% (95% CI 23–51) and 100% (95% CI 83.8–100), respectively.

The corresponding values for the hTERC FISH analysis were 70% (95% CI 47.8–85.5), 82.4% (95% CI 66–91.9), 70% (95% CI 49.9–90.1) and 82.3% (95% CI 69.5–95.2), respectively. The specificity and PPV of the DNA test were significantly improved by the hTERC FISH analysis (McNemar test;  $p < 0.0001$ ). ROC analysis for ac-

**Table 2.** hTERC amplification according to the cytological and histological diagnoses

Histology	Cytology, n (%)		
	ASCUS (n = 23)	LSIL (n = 25)	HSIL (n = 6)
NILM	0/5 (0)	0/3 (0)	0/0 (0)
CIN1	2/9 (22.2)	4/17 (23.5)	0/0 (0)
CIN2	5/7 (71.4)	1/2 (50)	0/0 (0)
CIN3	1/2 (50)	2/3 (66.7)	5/6 (83.3)

**Table 3.** Correlation between HPV-DNA test and hTERC FISH analysis

HPV-DNA test	hTERC FISH analysis		
	positive	negative	all
Positive	19 (95)	33 (97.1)	52 (96.3)
Negative	1 (5)	1 (2.9)	2 (3.7)
All	20 (37)	34 (63)	54

Values are expressed as n (%).

curacy showed an area under the curve (AUC) reaching 0.53 (95% CI 0.39–0.67) for the DNA test and 0.76 (95% CI 0.63–0.87) for FISH, respectively. Histological diagnosis, as the gold standard, showed an AUC of 1. Differences were statistically significant ( $p < 0.05$ ; fig. 2).

To investigate the relationship between HPV infection and hTERC gene amplification, we analyzed both tests as combined. Not all genetically abnormal cases were diagnosed as HPV positive, and not all HPV-positive cases showed hTERC gene amplification. There was oncogenic HPV infection in 95% (19/20) of positive hTERC cases (McNemar test;  $p < 0.05$ ; table 3). HPV-DNA/hTERC positivity increased along with the degree of cervical dysplasia ( $p < 0.001$ ).

### **Discussion**

The Pap test is unanimously accepted as the most cost-effective screening tool for detecting precancerous cervical disease. Thanks to the wide use of cytological testing, cervical cancer incidence is kept under control in the so-called industrialized countries, and the prevalence of relevant lesions in the population is currently very low.

In spite of this, cytological screening has some limitations, including the low sensitivity and the poor interobserver reproducibility of morphological interpretation. An audit of UK National Health Service Cancer Screening Programmes on cervical cancer found that CC occurred in 29% of women under 65 years of age, whose screening was up to date and in line with national guidelines [17]. ASCUS diagnosis, which is considered the borderline between clearly normal and clearly abnormal, is pathognomonic in this sense, underlying 38.8% of histologically confirmed high-grade lesions [18–21]. On the other hand, only 10% of cervical lesions are at a high risk for progression towards invasive cancer [22]. This fact is particularly true for low-grade cervical abnormalities, the management of which is not yet clearly defined, ranging from repetition of cytology at shorter intervals to immediate referral to colposcopy [18]. In summary, an LSIL diagnosis would often cause overtreatment and extensive costs [19], while patients with an ASCUS diagnosis would have a small but significant risk of progression toward high-grade lesions [20].

To improve the diagnostic accuracy of cytology, many tests have been evaluated, such as HPV-DNA test and p16<sup>INK4a</sup>. The American Society for Colposcopy and Cervical Pathology recommends to triage patients with ASCUS and LSIL with HPV-DNA assay [23, 24], but the transitory nature of HPV infection would confer a low specificity to the HPV-DNA test for detecting high-grade intraepithelial neoplasia or invasive cancer. For this reason, the scientific community is looking for a supplemental test that would bypass this issue and provide information on the regression or progression of cervical lesions.

Several factors, such as a loss of cellular inhibiting factors and the integration of the HPV genome into the host genome, can lead to the expression of E7 in the basal cell layers. The result is cellular transformation and a massive accumulation of p16<sup>INK4a</sup> in the cells [25]. The usefulness of the immunocytochemical detection of p16 in cervical samples has been analyzed in many trials, particularly as the triage test for ASCUS and LSIL. While the sensitivity of p16 in detecting underlying CIN2+ was similar to that of HPV testing, its specificity was confirmed to be higher [26]. On the other hand, a recent meta-analysis showed a substantial variability in the literature regarding the cut-off for positive p16 results [27]. This variability is probably due to the wide range of color-based qualitative (i.e. nuclear or cytoplasmic positivity and staining intensity) and quantitative parameters (i.e. the number of immunoreactive cells) which (singly or in combination) are usually used. The purely color-based approach to identify

abnormal cells in cervical smears using p16, is hampered by the fact that some normal endocervical, endometrial, intercalated, squamous metaplastic or atrophic cells may also display p16 immunoreactivity. The use of a 4-tiered classification score has been proposed [28]. However, due to the high variability when relying on p16 interpretation and despite the relatively low cost of this immunohistochemical assay (EUR 57 vs. 81.60 for DNA testing and EUR 164.55 for hTERC FISH according to the Italian NHS tariff), there is still disagreement about the use of p16 as a predictive marker in clinical practice.

Several studies postulated the essential role of 3q26 during the progression of cervical lesions to invasive cancer [24], but the application of molecular techniques able to identify hTERC gene amplification in a clinical setting is still evolving [29–33]. Our goal was to evaluate 3q26 hTERC FISH analysis on liquid-based cervical samples, in order to establish its accuracy as a predictive test and its feasibility for routine application.

Our study differs from previous reports [34, 35]. To our knowledge, this is the first time that hTERC FISH analysis has been performed as a reflex test on residual LBC specimens which have also been used for cytological diagnosis and HPV-DNA testing. Moreover, the inclusion of patients with varying degrees of cytological abnormalities made our setting different from the study by Ying et al. [36], who conducted their analyses in an HPV-DNA-positive population exhibiting ASCUS and LSIL.

The percentage of successfully hybridized samples was 100% in our study, considerably higher than in other studies [30–33]. In keeping with previous studies, we found a significant correlation (95%) between the HPV-DNA test and hTERC FISH. This finding would confirm the possible role of HPV in the amplification of the hTERC gene [34]. Like Branca et al. [35], we found a linear relationship between higher grades of CIN and the percentage of cervical samples in which hTERC amplification was found. Our data showed that extra copies of the hTERC gene were present in about 23% of CIN1, 66.7% of CIN2 and 72.7% of CIN3 cases, but were lacking in nonpathological tissue. Moreover, the hTERC analysis significantly differentiated the CIN2– from the CIN2+ cases. Branca et al. [35], on the other hand, evaluated the expression of hTERC on histological specimens by immunohistochemical assay, grading the nuclear staining into 4 categories. Immunohistochemistry demonstrates high feasibility in the clinical setting, but its use for hTERC assessment is sometimes difficult due to special conditions under which nondysplastic cells also stain positive. In such cases, the evaluation of hTERC-positive cells re-

quires subjective interpretation of both cellular and nuclear morphology, and would possibly lead to poor reproducibility of results.

Considering the cytological categories, we demonstrated that patients with an HSIL diagnosis had a significantly higher rate of hTERC gene amplification than patients with ASCUS and LSIL.

Among the ASCUS and LSIL cases, hTERC analysis enables significant differentiation between patients with CIN2+ and CIN2-, thereby improving the PPV of such cytological categories. These findings, comparable with previous studies, strongly support the potential clinical usefulness of this genetic test for the correct management of women with borderline/low-grade cervical abnormalities [36–40].

ROC curves, integrating sensitivity and specificity for a diagnostic test, would supply a measure of its accuracy. The AUC, in particular, has proved to be a powerful tool to measure the ability of a test to correctly classify patients with or without disease. In our study, the ROC curves demonstrated the higher accuracy of hTERC FISH for the detection of CIN2+ in comparison to that of DNA test.

We found that 87.5% of NILM cases tested HPV-DNA positive, even though the study population consisted of women with a mean age of 35 years. There are several possible explanations for this. First, there is the specific limitation of the HC2 technique; it does not include internal control for quality or performance. Thus, the presence of a very high load of nononcogenic HPV-DNA in the sample, as we found, would give rise to false-positive results for oncogenic genotypes by cross-hybridization [41]. This is certainly a considerable problem in populations with a high predominance of infections induced by these viral genotypes. Pathognomonic in this sense is the paper by Adams et al. [42], in which 12.2% of the cases with an ASC diagnosis revealed an oncogenic HPV infection by HC2 and a negative cervical biopsy result.

Another reasonable explanation for a negative biopsy result in women with oncogenic HPV infection would be the sampling error that occurred during colposcopic examination [42]. This hypothesis would underline the importance of the use of predictive markers during follow-up of HPV-positive patients. Finally, the substantial number of cases in our study that had a positive HPV-DNA test associated with a negative histological diagnosis might be also related to the natural history of the HPV infection itself. HPV infection can be transient, especially but not exclusively, in women  $\leq 30$  years of age. The probability of spontaneous resolution is high within the sec-

ond year [43]. About 43% of the patients in our study with a negative biopsy but an HPV-positive result were  $< 30$  years old, but most of them underwent biopsy within 1 year of the index HPV-DNA test. For all the reasons listed above, hTERC FISH analysis appears to be a promising discriminating tool, able to distinguish cervical lesions on the basis of their progression potential.

Finally, there are some considerations about the feasibility of the hTERC FISH test in clinical practice. hTERC FISH technology is similar to that used for Uro-Vision, which is widely utilized to analyze urine specimens. We strongly believe that advances in cervical LBC and automation during FISH procedure would allow for the routine use of hTERC analysis in clinical settings. In particular, we recommend both HPV and FISH testing on the same vial. In view of this, the ability to perform multiparametric analysis on the same cervical specimens would certainly lead to more effective CC prevention strategies.

In our opinion, the combination of the HPV-DNA test and hTERC FISH analysis can serve as an important application for the management of women with borderline/mild cervical abnormalities [36]. We are aware that our study had some limitations, such as the small number of cases. To obviate this drawback, further prospective investigations with long-term follow-up will be necessary, in view of the possible introduction of hTERC FISH analysis into decision-making algorithms [44–50].

## Disclosure Statement

The authors declare no conflicts of interest.

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