Gain of the 3q26 region in cervicovaginal liquid-based pap preparations is associated with squamous intraepithelial lesions and squamous cell carcinoma

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Abstract

Background. Chromosomal aberrations have been documented in cervical carcinomas, especially chromosome 3q. The human telomerase RNA gene (hTERC) is located in the chromosome 3q26 region, and its product, telomerase, is involved in the maintenance of chromosome length and stability. Uptregulation of telomerase is in general associated with tumorigenesis. In this study, cervicovaginal specimens were analyzed by fluorescence in situ hybridization (FISH) for gain of chromosome 3q26 containing hTERC, and FISH findings were compared with the cytologic and histologic diagnoses.

Methods. Slides prepared from 66 liquid-based preparations from cervical specimens with cytologic diagnoses of negative for squamous intraepithelial lesion or malignancy (NILM, n = 4), atypical squamous cells of undetermined significance (ASC-US, n = 15), low-grade squamous intraepithelial lesion (LSIL, n = 20), high-grade squamous intraepithelial lesion (HSIL, n = 24), or cervical squamous cell carcinoma (SCCA, n = 3) were analyzed for aberrations of 3q26 using a commercially available two-color FISH probe. The results of the cytologic analysis and those of concurrent or subsequent biopsies, when available, were compared with the FISH-detected 3q26 abnormalities. The Wilcoxon rank–sum test was used to assess associations between 3q26 gains and diagnoses.

Results. Gain of 3q26 was significantly associated with the cytologic diagnosis (p < 0.0001). Patients with HSIL or SCCA cytology diagnoses had significantly higher percentages of cells with 3q26 gain than did patients with NILM or ASC-US cytologic diagnoses.

Conclusions. FISH can be performed on cervicovaginal liquid-based preparations to detect gain of 3q26. Gain of 3q26 is associated with HSIL and SCCA. This test may be an adjunct to cytology screening, especially high-risk patients.

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Introduction

Cervical carcinoma is the most common gynecologic tumor in women worldwide. Developed nations have lower rates of morbidity and mortality than developing countries [1] largely because of effective screening programs [2]. Despite improved screening programs it is estimated that in the United States 11,150 women will develop cervical cancer in 2007 [3]. Although infection with the human papillomavirus (HPV) is considered the most significant factor in initiating cervical carcinogenesis, other genetic events are necessary for a cervical precancerous lesion to progress to malignancy [4]. Among the chromosomal abnormalities identified in cervical carcinoma, the most consistent aberration is gain of chromosome arm 3q, which is detected in approximately 70% of cervical carcinomas [5–10] Interestingly, the chromosome 3q26 region contains the human telomerase RNA gene (hTERC) [11].

Telomerase is involved in the maintenance of chromosomes by providing telomere stability and regulating telomere length.
Telomerase is composed of two main components, an RNA subunit (hTERC) that serves as a template for telomere addition and a protein subunit (hTERT) that acts to catalyze telomere synthesis [12]. Some tumors and tumor cell lines show upregulation of hTERC suggesting that hTERC transcription is upregulated during tumorigenesis [13].

Previous studies have analyzed the gain of 3q26 in cervical carcinomas, but it is unclear whether this genomic abnormality can be used to identify patients with cervical precancerous lesions who are at a higher risk of developing cervical cancer. In this study, we investigated the prevalence of 3q26 gain, as measured by fluorescence in situ hybridization (FISH), in routine liquid-based cytologic preparations from cervicovaginal specimens with a variety of cytologic diagnoses. In addition, we assessed associations between the FISH results and cytologic and histologic diagnoses.

Methods and materials

Cytologic specimens

The pathology database at The University of Texas M. D. Anderson Cancer Center was searched to identify all cases in which cytologic diagnoses were made from liquid-based preparations from cervicovaginal specimens, the abnormal cytologic diagnoses were confirmed histologically, and residual material was available from which an additional slide could be prepared for FISH analysis. This pilot study was approved by the institutional review board with a waiver of consent.

As part of the original diagnostic work-up, SurePath (TriPath Imaging, Inc., Burlington, NC) liquid-based preparations were stained using the Papanicolaou method, screened by a cytopathologist, and interpreted according to the Bethesda System [14] as follows: negative for intraepithelial lesion or malignancy (NILM, 4 cases); atypical squamous cells of undetermined significance (ASC-US, 15 cases); low-grade squamous intraepithelial lesion (LSIL, 20 cases); high-grade squamous intraepithelial lesion (HSIL, 24 cases); and cervical squamous cell carcinoma (SCCA, 3 cases). Fourteen of the 15 ASC-US cases had HPV DNA testing as part of the diagnostic work-up, as did 3 LSIL cases and 1 HSIL case. HPV DNA testing was performed using the Hybrid Capture II system (Digene Corporation, Gaithersburg, MD). The histologic diagnoses were obtained from the database and categorized as follows: negative; koilocytic changes; cervical intraepithelial neoplasia (CIN) I, II, and III; and SCCA.

Cervixcyte FISH

For this study, a slide was prepared using the SurePath technique and fixed with FISH fixative (3:1 methanol:acetic acid) for 30 min. The Cervixcyte probe panel (Cancer Genetics, Inc., River Vale, NJ) which consists of two probes: 3q26 (red) and the control, centromeric chromosome 7 (green). The 3q26 probe consists of six overlapping BAC clones that cover an 839-kb region that includes hTERC which encodes the RNA component of telomerase [15].

FISH testing was modified in cooperation with the manufacturer. The FISH-fixed slides were placed in preheated 2+ sodium saline citrate (SSC; pH 7.0) at 73 °C for 2 min, digested in a protease solution (0.5 mg/ml; pH 2.0) for 10 min at 37 °C, and fixed in 1% formaldehyde for 5 min at room temperature. After dehydration of the slides in a graded series of concentrations of ethanol, 10 μl of probe (pre-incubated at 96 °C for 5 min) was applied onto each slide, which was then coverslipped and sealed with rubber cement. To denature DNA, the slides were placed on a 75 °C preheated plate (HYBrite, Vysis, Downer Grove, IL) for 5 min and then hybridized overnight at 37 °C. After 16–18 h of hybridization, slides were washed in 45 °C preheated post-hybridization buffer (2× SSC/0.1% sodium dodecyl sulfate) three times for 5 min each and rinsed in distilled water. After air-drying (out of direct light), the slides were counterstained with 20 μl of DAPI/anti-fade solution and coverslipped.

Signal enumeration

FISH analysis was performed by a cytopathologist who has 8 years of experience in FISH analysis and who was blinded to the cytologic and histologic findings at the time of the evaluation. The slides were scanned using a Leica DMLB fluorescent microscope (Leica, Wetzlar, Germany) equipped with a 100-watt mercury lamp and Vysis single band pass filter set to detect DAPI, rhodamine (3q26), and FITC (chromosome 7) at 630×. The signals were evaluated by using a scanning method similar to that applied to the UroVysion [16]. The most atypical 25 cells were scored for abnormal signal numbers (loss or gain) of both 3q26 and centromeric chromosome 7 and recorded on a scoring sheet. An atypical cell was characterized by an enlarged nucleus with irregular nuclear membranes and/or a clearing around the nucleus. To avoid counting split signals as two signals, the distance between any two signals had to be at least the diameter of one signal for them to be counted as separate signals. A cell was scored positive for gain of 3q26 if more than two red signals were detected.
The primary statistical objective was to assess the association between the gain of 3q26 and the cytology or histology diagnosis. The proportion of 3q26 gains out of 25 cells was calculated. Gain of 3q26 was defined as greater than two signals within a nucleus. All statistics were based on the proportion of 3q26 gain. Descriptive statistics were calculated. Wilcox rank-sum tests were used to assess the association between 3q26 gain and cytology or histology diagnosis. All computations were carried out using SAS.

Results

The specimens analyzed were obtained from 66 women, ranging in age from 20 to 92 years (mean age, 46 years). Of the 4 NILM cases, 3 had a biopsy and all were negative for dysplasia and malignancy. Fourteen of 15 ASC-US cases had reflex HPV testing, and 5 of the 14 were positive for high-risk HPV subtypes. The 1 case of ASC-US without HPV testing had CIN I and koilocytosis on subsequent biopsy. Three other ASC-US cases had biopsies, and all showed koilocytosis. Of these, 2 were high-risk HPV positive, and 1 was HPV negative.

Of the 20 LSIL cases, 5 cases showed koilocytosis, 7 showed CIN I, and 8 showed CIN II–III on biopsy. Of the 24 cases of HSIL, 18 showed CIN II–III, 5 koilocytosis, and 1 SCCA on biopsy. All SCCA cytologic diagnoses were confirmed by biopsy.

Table 1 summarizes the 3q26 gain results by FISH analysis. None of the 4 NILM cases showed gain of 3q26 (Fig. 1), while 3 (13%) of the 15 ASC-US cases showed gain of 3q26. Of these, 2 were positive for HPV, but none of these had a biopsy. Of the 20 LSIL cases, 14 cases (70%) showed gain of 3q26 (Fig. 2) in 2–25 of the 25 cells analyzed, with a mean of 5.5 cells showing gain of 3q26. Only 1 LSIL case had >15 cells with gain of 3q26, and that case showed CIN I on biopsy. All 24 cases of HSIL (Fig. 3) and all 3 SCCA cases showed gain of 3q26. The number of cells with abnormal signal patterns varied in HSIL cases from 5 to 25, with a mean of 20. In all 3 cases of SCCA, all 25 atypical cells analyzed had abnormal 3q26 signal patterns (Fig. 4).

Statistical analysis showed that 3q26 gain was significantly associated with the cytologic diagnosis ($p < 0.0001$; Fig. 5). Patients with an HSIL or SCCA cytologic diagnosis had a significantly higher percentage of cells with gain of 3q26 than did patients with an NILM or ASC-US cytologic diagnosis. In cases diagnosed as LSIL on cytology, the percentage of cells with gain of 3q26 did not differentiate those histologically diagnosed as high-grade dysplasia (CIN II/III) from those diagnosed as low-grade dysplasia (CIN I) (Fig. 6).

Discussion

Gain of 3q26 was significantly associated with the cytologic diagnoses. Patients with a cytologic diagnosis of HSIL or SCCA had significantly higher percentages of cells with gain of 3q26 than did patients with an NILM or ASC-US cytologic diagnosis. This test may be an adjunct to cytology screening to detect patients with high-grade lesions.
SCCA of the uterine cervix arises from the progression of a precancerous lesion to carcinoma in situ and then to invasive carcinoma. The development of SCCA is strongly associated with high-risk HPV infection. Integration of high-risk HPV DNA into the host’s cellular genome results in constitutive expression of the oncoproteins E6 and E7, leading to deregulation of the cell cycle by inactivation of the \( p53 \) and \( pRB \) tumor suppress genes. Inactivation of these suppressor genes is thought to be crucial in initiating tumorigenesis [3,17,18]. However, the discordance between the high prevalence of HPV infection and the much lower prevalence of cervical cancer indicates that other genetic events are necessary for progression to malignancy [4].

Several authors have shown numerous genomic alterations in cervical carcinoma [5,7,8,19] Heselmeyer et al. [5] analyzed advanced-stage SCCA by comparative genomic hybridization and found recurrent losses and high copy numbers of chromosomes in these tumors. The most consistent chromosomal gain was at 3q in the aneuploid tumors, with the smallest amplicon mapped to chromosomal band 3q26-27. Heselmeyer et al. also found DNA copy number progression from normal cervical epithelium to invasive carcinoma, especially for chromosome 3, and proposed that gains of chromosome arm 3q play a crucial role in the progression of severe dysplasia/carcinoma in situ to invasive SCCA. Other studies on tissue sections have also shown that 3q gain is the most common chromosomal abnormality in high-grade dysplasias and SCCA [20,21].
The chromosome 3q26 region contains the hTERC gene that encodes for RNA unit of telomerase that maintains the length of telomeres through cellular divisions. When hTERC is over-expressed, the cells with critically short telomeres avoid undergoing apoptosis, potentially leading to tumorigenesis [13]. Another gene located at 3q26 is PIK3CA which encodes a catalytic subunit involved in the lipid-signaling pathology and it is associated with a number of cancer-associated functions including cell growth and apoptosis [22].

Previous studies have performed FISH analysis on cytologic cervicovaginal specimens and detected chromosomal aberrations in precancerous lesions [20,21,23,24]. Heselmeyer-Haddad et al. [25] analyzed 59 previously stained, routinely diagnosed Pap smears for 3q26 gains using a FISH probe that covered the region where hTERC is located. In their study, images were taken of the abnormal cells, and the x and y coordinates were recorded so that the same cells could be located again and analyzed for abnormalities of 3q26. Cases were considered positive for gain of 3q if more than 20% of the cells exhibited more than two signals (average number of cells counted not given). In our study, we analyzed abnormal cells on liquid-based cervicovaginal preparations for gain of 3q26 using a FISH probe similar to that used by Heselmeyer-Haddad et al. [25]; however, in our study the cells were analyzed differently. In each case, the most abnormal 25 cells were scored using a scanning method similar to that used with the UroVysion kit (Abbott Laboratories, Des Plaines, Ill) on voided urine specimens [14] The abnormal cells had irregular nuclei, and in some cases of LSIL with HPV changes, there was a slight clearing around the nuclei which could be visualized on the DAPI filter. A cell was scored positive for gain of 3q26 if more than two signals were present.

In another study by Heselmeyer-Haddad et al. [26], 57 liquid-based cervical preparations from patients with various precancerous lesions were analyzed for gain of 3q26. Similar to our study, the authors found that the percentage of cells with gain of 3q26 increased with the severity of dysplasia. In their study, a few normal, ASC-US, and LSIL cases showed increased 3q copy numbers, but at least 63% of HSIL cases had extra copies of 3q. It is interesting that 33% of cytologically negative smears with gain of 3q26 were from women who subsequently showed high-grade dysplasia on biopsy. None of the lesions that regressed had gain of 3q26, while those cases with high-grade dysplasia had this genetic aberration. The authors suggested that increased 3q copy numbers in cytologic specimens could serve as an independent screening test for HSIL. Our study on liquid-based preparations also showed significantly more cells with gain of 3q26 in cases of HSIL and SCCA. However, in cases of LSIL, gain of 3q26 did not differentiate those lesions that had CIN II–III on biopsy from those that did not.

A potential limitation of our study is that FISH analysis was performed on a different slide than the slide evaluated by cytology, even though both slides were prepared from the same vial. However, performing this FISH analysis on a previously Papanicolaou-stained slide is not optimal as destaining may leave remnants of stain that can interfere with interpretation of the FISH signals. In our study, only a few cases had both HPV and FISH testing. Further studies are needed to determine if sufficient material is present for performing both tests from one vial, especially in cases of atrophy. In addition, the original slide would no longer be available for cytologic review. Another limitation of our study was that concurrent biopsy results were not available in all cases, some were taken on subsequent clinic visits. Cervical biopsies taken at the same time as the cytology specimens are optimal for confirming the degree of dysplasia. However, this will probably not resolve all the discrepancies since previous studies correlating diagnoses of concurrently obtained cytologic and histologic specimens have shown concordance in only 74.4% of cases [27]. Furthermore, it is well known that there are sampling and interpretative errors by both methods in diagnosing cervicovaginal samples. Finally, our study set is small due to cost constraints, but it was designed as a pilot study containing various squamous abnormalities with a control group.

In conclusion, FISH can be performed on liquid-based preparations from cervicovaginal specimens to detect gain of 3q26, which contained the locus encoding for hTERC. The method used to score the FISH for gain of 3q26 is similar to that used for UroVysion which is already utilized in some laboratories for analyzing urine specimens, and so the widespread use of FISH to detect gain of 3q26 in cervical specimens seems feasible. The 3q26 FISH analysis will probably not substitute or be a replacement for HPV testing which is primarily performed in cases of ASC-US. Potential clinical usefulness of the 3q26 FISH would be to identify those cases of LSIL and borderline HSIL that have a more aggressive biologic behavior or an adjunct to cytology screening, especially in high-risk patients. However, more studies are needed to determine the sensitivity and specificity of this test and its application in screening.

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References


